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The Effects of Temperature on the Cardiac System of the American Lobster,

Homarus americanus

An Honors Paper for the Program of Neuroscience

By Elizabeth Ann Owens

Bowdoin College, 2014

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Abstract

The American lobster, *Homarus americanus*, inhabits a large oceanic range spanning from Labrador, Canada to North Carolina, USA. This geographic range varies in temperature by as much as 25°C, and daily temperature fluctuations of up to 12°C may occur at a single location depending on season, water depth, and tides. The cardiac system of the lobster is sensitive to these temperature changes, and has been shown to adjust its functioning over a large temperature range. A previous study showed that various functional parameters respond differently to temperature changes, but a stable cardiac output can be maintained over the range of 2-20°C. The current study showed that the effects of temperature were exerted primarily through changes in the lobster heart central pattern generator, the cardiac ganglion. Similar patterns of change were seen in both semi-intact hearts and isolated cardiac ganglion preparations in response to increasing temperature. Specifically, with increasing temperature, the burst frequency showed a biphasic pattern in which frequency initially increased, then decreased rapidly at high temperatures. The burst duration, duty cycle, and number of spikes per burst generally decreased with increasing temperature, and spike frequency increased over the entire temperature range. Semi-intact hearts and isolated cardiac ganglia showed similar “crash” patterns, characterized by complete loss of function at high temperatures and complete recovery of function when temperature was returned to baseline. Feedback in the semi-intact heart provided some stabilization of bursting activity, but it did not provide the expected protection from high temperatures. The isolated CG had a significantly higher crash temperature than did the semi-intact system. This discrepancy in crash temperatures may be explained by considering factors at the level of the muscle and neuromuscular junction (NMJ), such as stretch and nitric oxide (NO) feedback and the balance of facilitation and depression at the NMJ. Stimulated preparations showed defacilitation of contraction amplitude at high temperatures despite the maintenance of constant burst parameters of stimulation. Therefore, several factors contributing to the relatively low crash temperature of the intact system may be a shift in the balance of facilitation and depression at the NMJ, a depression in ganglion function due to the release of NO by the muscle, or a combination of the two mechanisms

Chapter 1

Introduction

1.1 Central Pattern Generators and Modulation

Central pattern generators (CPGs) are neural networks that control rhythmic, patterned behaviors. These networks contain neurons that spontaneously produce periodic bursts of electrical activity that drive the patterned behavior (Delcomyn 1980). The patterned behaviors driven by CPGs are diverse, and include respiration, chewing, intestinal movements, heartbeat, and various forms of locomotion (such as flying, swimming, and walking). These CPGs have been studied in a wide variety of animals, such as snails, locusts, crabs, frogs and cats (Ayali & Lange 2010, Delcomyn 1980, McCrea & Rybak 2008, Yamaguchi *et al.* 2008). Many of these systems are highly complex and involve large numbers of neurons, some of which may participate in multiple circuits at different times (Harris-Warrick & Marder 1991). Some invertebrate CPGs, however, are simpler and easier to study: they are often anatomically isolated in decentralized nervous systems, consist generally of a small number of neurons, and are easily manipulated *in vitro* (Cooke 2002). These simple systems have therefore been the focus of research aimed at providing an understanding of the general mechanisms of CPG and motor system function, which may be applied to more complex systems in the future.

A defining feature of central pattern generators is their ability to produce rhythmic firing in the absence of any sensory input (Delcomyn 1980). However, CPGs are often responsive to sensory inputs and internal feedback that provide information to the system and allow for adjustments in motor output in response to a variety of conditions. This modulation often provides a stabilizing effect on the motor output in response to environmental perturbations. Modulation may occur at multiple levels of the system, including at the level of individual

neurons (by affecting intrinsic properties like ionic currents and synaptic connectivities), the neuromuscular junction, the muscle, or sense organs (Dickinson 2006, Fort *et al.* 2007a, Fort *et al.* 2007b, Harris-Warrick & Marder 1991). Each component of the CPG-effector system is subject to multiple modulatory factors, which must work cohesively to maintain stereotyped network outputs in diverse conditions.

1.2 The Lobster Cardiac Ganglion

One simple system used in CPG research is the lobster cardiac ganglion. The lobster heart is neurogenic: the heartbeat is driven by a pattern generator called the cardiac ganglion (CG), which produces rhythmic, patterned outputs that drive regular muscle contraction (Cooke 2002). The CG is a relatively simple network consisting of only nine neurons located on the inner dorsal wall of the heart (Figure 1a). There are four small, posterior interneurons termed pacemakers that spontaneously produce rhythmic bursts. These four pacemakers synapse onto the five larger, more anterior motor neurons (Figure 1b). The motor neurons innervate muscle fibers through the neuromuscular junction and drive the muscular contractions of the heartbeat (Cooke 2002, Hartline 1979, Mayeri 1973). In addition to chemical synapses, the ganglion cells are also electrically coupled, allowing for the maintenance of synchronous firing (Mayeri 1973).

The firing activity in the ganglion is dependent upon several distinct morphological regions of the neurons. Ganglion neurons have inactive, electrically-unexcitable soma and proximal axons, which produce slow, sustained, and regenerative driver potentials. The depolarizing driver potentials initiate spiking impulses along the distal axon by stimulating a train of action potentials (Hartline 1979). This train of action potentials makes up a burst, and may be measured through extracellular axonal recordings. It is possible to characterize the driver

potentials due to the physical isolation of the inactive and excitable regions. Driver potentials have a rapid depolarization with a rounded peak and falling shoulder, followed by a faster repolarization leading to hyperpolarized after-potentials (Cooke 2002). This pattern results from the interaction of an inward Ca^{2+} -current (I_{Ca}) and three outward K^{+} currents: a transient current (I_{A}), a slowly-inactivity current (I_{K}), and a calcium-dependent current (I_{KCa}) (Cooke 2002; Ransdell *et al.* 2013). The shape and pattern of these driver potentials depend on the balance of these various channel kinetics.

The electrical activity of the cardiac ganglion is transformed into the muscular contractions of the heartbeat at the neuromuscular junction. The cardiac ganglion neurons innervate the muscle at multiple locations by releasing neurotransmitter to post-synaptic receptors on the muscle. This receptor activation triggers depolarization of the muscle, leading to calcium influx and ultimately muscle contraction. Each burst of electrical impulses generates multiple excitatory junction potentials (EJPs) in the muscle that, in combination, produce the rhythmic heartbeat. The shape of the driver potentials underlying the ganglion signal is important for the efficient pumping of the heart. The fast rise in depolarization insures a rapid and coordinated start of muscle contraction, and the plateau insures maintained muscle shortening throughout the burst (Anderson & Cooke 1971). The transformation from neural signal to muscle contraction is complex and depends on many factors: presynaptic neuronal factors such as membrane potential and calcium dynamics; post-synaptic receptors and channels; and contractile properties of the muscle, including initial length and contractile force of the fibers (Williams *et al.* 2013). All of the variables involved in the production of neural signals and motor outputs provide physiological targets for modulation of the CPG-effector system at every level.

1.3 Feedback and Modulation in the Lobster Heart

The lobster heart is affected by feedback systems and external modulation. The heart contains both positive and negative feedback systems that affect ganglion functioning. It has been shown that during normal heartbeat contractions, nitric oxide synthase is present in the muscle, suggesting that the muscle produces nitric oxide during contractions. NO is able to diffuse to the cardiac ganglion, where it can produce a decrease in burst frequency of the ganglion and a consequent decrease in the contraction amplitude of the muscle. There is some debate over this mechanism, as perfusion with NOS inhibitors only increases heart rate in individuals with slow resting heartbeats (Goy 2005, Mahadevan *et al.* 2004). However, it is generally assumed that the level of NO in the system provides information to the ganglion about current muscle contractions and allows for adjustments to current conditions.

A second mechanism of feedback within the lobster heart relies on stretch. *In vitro* preparations require perfusion in order to maintain cardiac activity. While some suggest that the perfusion is necessary for proper oxidation of the tissue, it is also possible that perfusion artificially mimics the natural stretch experienced in the body cavity (Kuramoto & Ebara 1984, Kuramoto & Ebara 1985, Wilkens 1993). It is hypothesized that this stretch sensitivity is mediated through dendritic processes of the cardiac ganglion that are sensitive to the stretch of the muscle. Increased stretch of the heart often results in increased burst frequency, suggesting a system of positive feedback (Garcia-Crescioni *et al.* 2010).

In addition to these feedback mechanisms, the lobster heart is responsive to external modulation. Much of the existing research has focused on the identification of peptides and other molecules that have excitatory and inhibitory effects on the cardiac system. There are dozens of peptides that act alone and in concert at every level of the CPG-effector system (Dickinson 2006,

Dickinson *et al.* 1997, Harris-Warrick & Marder 1991, Stevens *et al.* 2009). However, the effects of these peptides are limited to the locations containing specific receptors. There are certain environmental factors that may provide a global perturbation to the entire system by affecting every level simultaneously and most likely in different ways.

Temperature is one global factor that influences the reaction rates of all cellular processes, including those involved in neuronal excitability and synaptic transmission. While temperature affects all processes, it may affect each process differently, such that the various circuit components respond differently to temperature perturbations. Overall, the frequency of CPG motor outputs increases (either linearly or exponentially) in response to temperature. This pattern is seen across many species and CPG systems, including crab digestion, locust ventilation, and frog vocalizations (Rinberg *et al.* 2013, Robertson 2004, Yamaguchi *et al.* 2008). The increase in output frequency is often described using a Q_{10} value, which measures the rate of change in response to increasing the temperature 10°C . Q_{10} s differ greatly depending on the system, but often range from 1 (no dependence on temperature) to almost 4 in mouse respiration (respiration almost quadruples when temperature is raised 10°C) (Robertson & Money 2012).

While temperature consistently increases the frequency of CPG outputs across various systems, it can have a variety of effects on underlying system parameters. The eventual failure of CPG systems at high temperatures may be due to the dissociation of underlying parameters differentially affected by temperature. The nervous system is particularly vulnerable to the effects of temperature due to the balance of ion channel kinetics needed to produce neural signals. CPGs depend upon the precisely-timed openings and closings of many ion channels, and these timings may be thrown off in high temperature environments (Robertson & Money 2012).

Failures in nervous tissue often result in increased extracellular potassium concentrations, which are restored to normal values upon recovery (Money *et al.* 2009, Robertson 2004). Temperature may also exert effects at the level of the neuromuscular junction and muscle fibers in CPG-effector systems, which contributes to the change in system output (Prosser & Nelson 1981).

1.4 Temperature in the Lobster Environment

Temperature is a particularly salient feature of lobsters' environments. Lobsters are poikilotherms which cannot regulate their body temperatures, so they must be able to adjust internal processes in response to changes in temperature that they cannot control. It is well-documented that lobsters alter behavior in response to temperature changes; they show a general preference for water of about 16°C in gradient experiments (Reynolds & Casterlin 1979). Lobsters also migrate seasonally, presumably to maintain temperatures that are developmentally and reproductively beneficial (Cowan *et al.* 2007). Ovarian maturation requires cold temperatures (below 8°C), while late stages of embryonic development require higher temperatures (Cowan *et al.* 2007). Therefore, the complete reproductive cycle of the lobster requires a complex pattern of temperature changes that relies on appropriate behavioral responses.

In addition to eliciting migratory behavior, temperature also has more direct physiological effects on many lobster systems, including the heart. The American lobster lives in an environmental range spanning from Labrador, Canada to North Carolina, USA, which encompasses a 25°C temperature range. Depending on water depth, seasons, and tides, lobsters may encounter daily variations of up to 12 to 15°C (Jury & Watson 2000; Worden *et al.* 2006). The temperature of the pericardial sinus matches the surrounding water temperature of an intact

lobster, meaning the heart is directly exposed to temperature and any fluctuations that may occur (Worden *et al.* 2006). In addition, lobsters can detect fluctuations with high resolution, and a temperature change as small as 0.15°C results in altered cardiac activity (Jury & Watson 2000). Although hearts tested under laboratory conditions are usually exposed to faster rates of temperature change than actually occur in the wild, laboratory heart rate data matches data taken from lobsters in the wild. This suggests that changes seen in the laboratory are faithful representations of physiological responses that actually occur in the wild (Worden *et al.* 2006).

This high sensitivity to changes in temperature is particularly concerning considering the recent rise in ocean temperatures. A study of lobsters in Long Island Sound found that the large die-off of lobsters, particularly egg-bearing females, was correlated with higher bottom water temperatures of around 20°C (Howell *et al.* 2005). In addition, warm-acclimation does not protect animals from heat stress, and may paradoxically make them more vulnerable. While warm-acclimated lobsters can survive to slightly higher temperatures than their cold-acclimated counterparts, they live relatively closer to the upper lethal limit temperature, making them more vulnerable to small increases in temperature (Camacho *et al.* 2006, Stenseng *et al.* 2005). This upper lethal limit temperature in several closely-related species corresponds closely to the upper limit of heart function, suggesting that survival at high temperatures is limited by cardiac function (Somero 2005).

1.5 Studying Temperature Effects in the Lobster Heart

The effects of increasing temperature on the entire cardiac system have been documented (Worden *et al.* 2006). Cardiac activity is altered in response to increased temperature in stereotyped ways. As water temperature increases, heart rate increases both *in vivo* and *in vitro*.

This is accompanied by an increase in the rates of muscle contraction and relaxation. At the same time, increasing temperature results in decreased heartbeat strength and stroke volume. These parameters counteract each other in a compensatory manner that results in a stable cardiac output over the range of 2 to 22°C. Beyond this temperature, function quickly deteriorates, and the heart eventually ceases to beat (Rinberg *et al.* 2013; Worden *et al.* 2006). The differing temperature-dependencies of heart beat frequency and contraction reveal that these parameters are independently controlled. The individual components of the cardiac system, as discussed above, are differentially affected by temperature, and thermal stress often causes a dissociation of patterned behavior that leads to crashes of the entire system (Tang *et al.* 2012).

While the cardiac responses to temperature changes are well known, it is still unknown how the cardiac system regulates these responses. Temperature most likely affects every component of the cardiac CPG-effector system in different ways. The question then arises: how do these changes interact to produce a stable output over a wide temperature range? In addition, new computational modeling and experimental work has shown that stereotyped network performance can result from different combinations of varying synaptic and intrinsic properties of network components. For example, relative ion channel densities in a particular neuron may vary 3- to 5-fold across individuals but still produce the same firing patterns (Goaillard *et al.* 2009, Marder 2011, Marder & Goaillard 2006, Prinz *et al.* 2004). Not only are all underlying parameters of cardiac function differentially affected by temperature, but the underlying parameters may differ widely by individual. How, then, do individuals respond in stereotyped manners to increases in temperature?

The current study investigated the effects of temperature on the lobster cardiac system. We began by characterizing the effects of temperature on the whole heart. We then characterized

the effects of temperature on the isolated component parts of the system (the isolated cardiac ganglion and the isolated muscle and NMJ). The responses of the system to increases in temperature were mediated primarily through changes in ganglion functioning, but the muscle and NMJ contributed to changes in the intact system as well.

Chapter 2

Methods

2.1 Animals

American lobsters were purchased from local seafood retailers in Brunswick, ME. They were maintained in recirculating natural seawater aquaria at 10-12°C, and were fed squid weekly. It was closely noted how long each lobster was acclimated to these conditions before dissection. A minimum acclimation period of two weeks was desired to minimize individual and seasonal variation, as most regulatory processes reach a stable level by this time (Tang et al., 2012; Camacho et al., 2006; Prosser & Nelson 1981, Worden et al., 2006). However, due to availability, actual acclimation period ranged from 1 to 25 days in laboratory tanks prior to dissection.

2.2 Semi-Intact Heart Preparation

Lobsters were anaesthetized on ice for 30-60 minutes before dissection. The heart was removed along with the overlaying section of the dorsal thoracic carapace and pinned ventral-side-up in a Sylgard-lined dish. The heart remained attached to the carapace to maintain the natural stretch present in the intact animal. The heart was covered in cold physiological saline (ACS grade: NaCl 479.12, KCl 12.72, CaCl₂ 13.67, MgSO₄ 20.00, Na₂SO₄ 3.91, Trizma base 11.45, maleic acid 4.82 (in mmol), pH 7.45).

The heart was cannulated through the posterior artery and was continuously perfused with cold physiological saline. The flow rate of perfusion was maintained at 2.5 ml/min to preserve the stretch necessary for the heart to continue beating outside of the intact animal. A second perfusion line ran along the side of the heart to maintain the temperature on the exterior

of the heart. A temperature probe (Warner Instruments, Hamden, CT, USA) was fitted alongside the bottom of the perfusion tubing and lay directly on the exterior ventral wall of the heart during the entire experiment. The temperature was continuously monitored and maintained throughout the preparation setup at a baseline of 7-9°C through an in-line temperature control system (CL-100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments).

To record the muscular contraction of the heartbeat (Figure 2a), the anterior arteries were tied with 6/0 Suture Silk to a Grass FT03 force-displacement transducer (Astro-Med, West Warwick, RI, USA) at an angle of approximately 30 degrees from the horizontal plane. The anterior arteries were stretched to produce a baseline tonus of 2g needed to mimic the stretch in the intact animal. The contraction output was amplified with a Model 410 Brownlee Precision Instrumentation Amplifier and recorded using Spike2 v6.09 software (Cambridge Electronic Design, Cambridge, UK). A small hole was then cut into the ventral heart wall slightly posterior to the ostia to expose the cardiac ganglion. A small portion of one of the anterolateral nerves was sucked into a suction electrode to record the extracellular electrical output of the ganglion while still connected to the heart muscle (Figure 2a). The electrode signal was recorded and amplified with a Model 1700 A-M Systems Differential AC Amplifier and a Model 410 Brownlee Precision Instrumentation Amplifier. The semi-intact heart was allowed to equilibrate for one hour prior to testing.

2.3 Isolated Cardiac Ganglion (ICG) Preparation

The cardiac ganglion was isolated from each heart used in the semi-intact preparation, allowing for a paired analysis with each heart acting as its own control. The heart was removed from the dorsal carapace and pinned down in a Sylgard-lined dish and covered in cold

physiological saline. The ventral wall of the heart was opened with a medial cut, and the muscle was pinned back to expose the ganglion. The ganglion, including the main trunk, the anterolateral nerves, and the posterior section containing the pacemaker cells, was removed from the surrounding tissue. The ganglion was pinned in a Sylgard-lined dish and covered in cold physiological saline (Figure 2b).

Extracellular recordings were made using either stainless steel pin electrodes or a suction electrode. For the former method, a Vaseline well was made around one of the anterolateral nerves of the ganglion (preferably not the one suctioned in the semi-intact preparation). One pin electrode was put into the well to record the electrical activity of the motor neuron axons present in the anterolateral nerve. Vaseline wells sometimes melted at high temperatures, so the latter suction electrode method was used in later experiments. This method involved suctioning up one anterolateral nerve, as in the semi-intact preparation, and ensuring the suction was watertight. The output was amplified and recorded using the same equipment as described above.

2.4 Stimulated Heart Preparation

Some hearts were used for three distinct preparations, the first of which was the semi-intact preparation described above. Then, the ganglion was removed while the heart muscle remained attached to the dorsal carapace. The ventral wall of the heart was cut open anteriorly from the dorsal abdominal artery to the ostia, leaving the anterior half of the heart intact. The ganglion was dissected out, and one anterolateral nerve was cut fairly short in order to leave a visible nerve ending easily accessible for stimulation (the isolated ganglion was prepared and tested as described above). Care was taken not to damage the muscle surrounding the CG anterolateral nerves during dissection. The muscle was once again tied to a force transducer and

recorded as described above. The severed nerve ending was stimulated using a suction electrode (Figure 2c). Impulse trains containing spikes of 0.5 ms duration (60 Hz frequency), with a burst duration of 300 ms at a burst frequency of 1 Hz were applied to the nerve ending. Each train contained 15 bursts, and the heart was left unstimulated for 60 seconds between trains to avoid the degraded muscle activity associated with continual stimulation. Electrical impulses were generated using the Micro 1401 data acquisition board and controlled by a custom Spike2 sequencer file (and injected through a Model 1700 A-M Systems Differential AC Amplifier). Both saline perfusion tubes were set to flow into the intact portion of the heart, over the muscle and nerve ending where the heart receives external stimulation. Muscle contractions and injected current were recorded using the same instrumentation and software as the other preparations.

2.5 Temperature Experiments

For each preparation, baseline functioning at 7-9°C was recorded for ten minutes. Then, using the Warner temperature system, the temperature of the saline pumped through or over the heart or ganglion was increased by 1.5°C every two minutes, stepwise. Later experiments used an automatically-controlled temperature program that increased the temperature smoothly instead of stepwise, at the same rate of change. The temperature of the saline in semi-intact and ICG preparations was increased until the system reached its “crash temperature.” The “crash temperature” was defined as the temperature at which the ganglion entirely stopped bursting and the muscle stopped contracting for at least 30 seconds, or at which there were three consecutive periods greater than 10 seconds. Previous experiments have shown that this criterion is indicative of heat stress, and may be used to estimate the upper limit of cardiac function. Importantly, ganglia and muscle function must have been able to recover from this peak temperature in order

for it to be defined as a “crash;” if no recovery was evident, then the peak temperature was considered lethal (Camacho et al., 2006; Tang et al., 2012).

For the stimulated preparation, the temperature was increased 1.5°C (stepwise) after each set of 15 injected current pulses, until the temperature reached the crash temperature of the semi-intact preparation. The isolated muscle does not show “crashing” behavior as defined in the semi-intact and ICG preparations. Preliminary experiments showed that if the stimulated muscle was allowed to reach a temperature where muscle contractions stopped entirely, activity could not be recovered upon return of temperature to baseline values. Therefore, the maximum temperature for stimulated preparations was based on the crash temperature of the semi-intact heart.

Once at the crash temperature, the saline was returned to 8°C, decreasing by 2°C/2min, either stepwise or in an automatically-controlled smooth ramp. The system was then allowed to run at baseline temperature for at least 20 minutes to record a recovery period.

2.6 Data Analysis

Recordings of ganglion bursting activity in semi-intact and ICG preparations were analyzed for burst frequency, burst duration, duty cycle (defined as the burst duration over the period), number of spikes per burst, and spike frequency. Recordings of muscle contractions in semi-intact and stimulated preparations were analyzed for contraction amplitude.

Temperature measurements were rounded to the nearest whole number to allow for grouped analysis. The “baseline temperature” was defined as the starting temperature for the experiment, and was usually 8°C. The “initial value” for each parameter was the average value at the baseline temperature. The “crash temperature” was defined as above. Averages and standard

deviations were calculated using all bursts at each rounded temperature for individual preparations. Coefficient of variation values were calculated from these individual average and standard deviation values. Individual preparations varied in the actual parameter values; for example, at baseline temperatures, some hearts burst at a frequency of 0.7 Hz, while others burst at a frequency of 0.3 Hz. To control for these differences, data were normalized so the initial parameter value (the average at the lowest temperature recorded) equaled 100%. Averages and standard deviations in combined data were calculated using the individual hearts' averages at each rounded temperature. Data from multiple hearts could then be compared without considering individual differences in the absolute parameter values. One isolated ganglion had a crash temperature 10°C higher than any other preparation, so it was not used in analysis.

Chapter 3

Results

3.1 Acclimation Period and Crash Temperature

Previous research shows that acclimation of lobsters for at least 2 weeks before dissection minimizes individual and seasonal variation (Camacho et al., 2006; Worden et al., 2006). The seasonal variability in water temperatures may shift the thermal limits of cardiac functioning, with lobsters in warm water able to maintain heart function at higher temperatures than those in cold water (Camacho *et al.* 2006). We attempted to acclimate all lobsters at 10-12°C for a minimum of two weeks. However, some lobsters were acclimated for a shorter time period due to availability. Lobsters dissected in the summer months (June-August) had no significant correlation between length of acclimation and crash temperature (Figure 3a). These lobsters were likely caught in waters comparable in temperature to the acclimation tanks used in the laboratory (10-12°C). There was a significant correlation between length of acclimation period and crash temperature for lobsters dissected during the academic year (September-February, Figure 3b). Lobsters with longer acclimation periods had higher crash temperatures in both semi-intact ($r(12) = .5813$, $p < 0.05$) and ICG ($r(12) = .6322$, $p < 0.05$) preparations than those with short acclimation periods. These lobsters were likely caught in waters colder than the laboratory acclimation tanks (National Oceanographic Data Center).

3.2 Characterization of Crashing Behavior in the Semi-Intact Heart

Crashing of the cardiac system was defined as the complete loss of function at high temperatures (loss of ganglion bursting and muscle contraction) for at least 30 seconds and by the recovery of function when the temperature was returned to baseline (Figure 4). The absolute

value of the crash temperature varied by individual heart, but the pattern of loss and recovery of function was seen across all hearts.

The functioning of the ganglion changed dramatically in response to increasing saline temperatures (Figure 5). These patterns of change were seen across all hearts, and will be discussed in detail below. Briefly, the burst frequency increased as temperature increased, until high temperatures (close to the crash temperature), at which the frequency became uneven and finally dropped to zero. The burst duration decreased as temperature increased. This was correlated with both a decrease in the number of spikes per burst and an increase in the spike frequency. The duty cycle decreased with increasing temperature, due to the relative rates of change of the burst duration and frequency. Finally, the amplitude of the extracellularly-recorded action potentials increased with increasing temperature.

Burst frequency in semi-intact hearts followed a stereotyped pattern of change with increasing temperatures: the burst frequency increased steadily up to about 10°C below crash temperature, after which the frequency decreased quickly until the ganglion finally stopped bursting. While each heart showed the same crash pattern, there were individual differences in the actual burst frequencies, the crash temperatures, and the amount of variation in the data (Figure 6a). For example, two hearts began with the same burst frequency, but reached different peak frequencies at different temperatures. A heart that started with one of the lowest burst frequencies (about 0.3 Hz) actually had one of the highest crash temperatures. Some hearts almost doubled in frequency at their peak, while others increased only slightly over baseline values.

In order to control for these individual differences, the data were normalized so the initial average burst frequency at baseline temperature equaled 100%. This allowed for the analysis of

the general patterns of change in response to increasing temperatures. The combined data showed the same pattern as the individual preparations – the burst frequency increased over approximately the first half of the temperature ramp, then decreased at higher temperatures (Figure 6b). The rapid decrease in frequency as a function of temperature near the crash temperature was obscured by individual differences in absolute crash temperature; the pattern can be seen more clearly when the crash temperatures for each heart are aligned (Figure 6c). The hearts reached an average of 142% of the initial value at peak frequency, which occurred on average 10°C below crash temperature. There was significant individual variation, with hearts ranging from 121% to 428% of the initial value at the peak. One preparation never increased in frequency and began to decline immediately upon temperature increase.

Burst duration steadily decreased in response to increasing temperatures (Figure 7). Individuals varied in the absolute burst duration; one heart had an initial burst duration of only 0.25 seconds, while another began with a duration of almost 0.5 seconds (Figure 7a). However, each individual reliably showed a decrease in duration over most of the temperature range (Figure 7b). Twenty four of the 30 hearts began to decline in duration immediately upon temperature increase. However, 6 of the 30 hearts increased slightly in burst duration at first, to an average of 132% of the initial burst duration. The hearts had decreased to an average of 32% of the initial burst duration by the time they reached their crash temperatures (before the bursts ceased entirely).

As temperature increased, the duty cycle remained relatively constant at first and then decreased slowly (Figure 8). The duty cycle is directly proportional to both burst duration and burst frequency, and so is dependent upon the relative changes in these two parameters. The stability of duty cycle over the beginning of the temperature ramp was therefore due to the

opposing increase in frequency and decrease in burst duration. The subsequent decrease in duty cycle was due to the fast decrease in burst duration and the slow increase (and later decrease) in burst frequency. This dependence on multiple burst parameters resulted in high individual variation in the pattern of duty cycle changes (Figure 8a). 18 of the 30 hearts decreased in duty cycle immediately, while 12 of the 30 hearts increased in duty cycle slightly at first, to an average of 112% of the initial value. At the crash temperatures, the hearts reached an average of 12% of the initial value.

The decrease in burst duration over the temperature range was correlated with a decrease in the number of spikes per burst ($r(23)=0.7822$, $p<0.0001$). The number of spikes per burst initially increased slightly, but then decreased steadily over the rest of the temperature range (Figure 9). On average, the number of spikes per burst peaked at 124% of the initial value, and reached 30% of the initial value at the crash temperature.

The decrease in burst duration was also correlated with an increase in spike frequency ($r(23)=-.09873$, $p<0.0001$). The spike frequency increased over the entire temperature range (Figure 10). On average, the spike frequency reached 380% of the initial value at the crash temperature, although there was significant individual variation ranging from 182% to 960% of the initial value at the peak.

Spike amplitude also increased with increasing temperatures in all preparations. This increase in amplitude was not quantified, but was obvious in recorded data (Figures 4 and 5). The extracellular ganglion recordings measure electrical activity of all motor neuron axons present in the nerve. Therefore, it is unclear if the increase in amplitude was due to an actual increase in action potential amplitude or to other factors affecting the extracellular recordings.

In addition to electrical ganglion activity, the response of the physical muscle contraction to increasing temperature was analyzed (Figure 11). The contraction amplitude increased slightly at first, reaching an average peak of 110% of the initial value around 12°C. The amplitude then decreased to an average of 20% of the initial value by the crash temperature (Figure 11b). There was significant individual variation in actual contraction amplitude (Figure 11a), with hearts ranging from 0.6 to 3.3 grams of force at baseline temperatures. Though most hearts increased modestly in amplitude, one heart doubled its contraction amplitude at its peak. Three of the 26 hearts did not increase at all; contraction decreased immediately upon temperature increase. Importantly, all of the hearts used for data analysis recovered at least 50% of the initial contraction amplitude upon return to baseline temperature. This criterion was important for distinguishing between crash temperature, which caused reversible cessation of function, and lethal temperature, which resulted in permanent damage to the muscle.

3.3 Characterization of Crashing Behavior in the Isolated Cardiac Ganglion

The cardiac ganglion produced similar patterns of crash in the isolated preparation as in the semi-intact preparation (Figure 4b). Once again, the crash behavior was defined as the complete loss of ganglion activity at high temperatures with fully recovered function upon return to baseline temperatures. The same functional parameters were analyzed for the isolated cardiac ganglia as for the semi-intact ganglia, and the same patterns were noted in response to increases in temperature. Generally, burst frequency increased then decreased sharply around the crash temperature. In contrast to what happened in the whole heart, burst duration actually increased slightly in the ICG before decreasing over most of the temperature range. This pattern was also

seen for duty cycle and number of spikes per burst. Similar to the whole heart, the spike frequency increased over the entire temperature range, as did spike amplitude.

Burst frequency increased with increasing temperature, and then rapidly decreased as the hearts began to crash (Figure 12). As in the semi-intact hearts, isolated ganglion frequency varied significantly by individual (Figure 12a). Individual ganglia varied in baseline frequency from 0.23 Hz to almost 1.1 Hz. In order to compare the pattern of crashing among ganglia, these individual differences were minimized by once again normalizing data so the average frequency value at baseline temperature equaled 100% (Figure 12b). The actual crash pattern is not clear on an absolute temperature scale due to individual differences in crash temperature, so the data were also plotted with crash temperatures aligned (Figure 12c). On average, the burst frequency reached 152% of the initial value at 6°C below the crash temperature. Therefore, the decrease in frequency occurred over a narrower temperature range in ICG preparations than in semi-intact hearts (6°C compared to 10°C).

The burst duration on average slightly increased before decreasing over most of the temperature range (Figure 13). Sixteen of the 26 isolated ganglia increased in burst duration to an average of 169% of the initial value, with individual values ranging from 105% to 239% at peak duration. In the other 10 ganglia, burst duration decreased immediately, which more closely followed the pattern seen in semi-intact hearts. At crash temperatures, the burst duration reached an average of 30% of the initial value.

The duty cycle had the same pattern as the burst duration: it increased slightly at first, and then decreased over the rest of the temperature ramp (Figure 14). The duty cycle is directly proportional to the burst duration, so the initial increase in duty cycle corresponds to the initial increase in the burst duration. The decrease in duty cycle then corresponds to the decrease in

burst duration, which must have occurred at a faster rate than the rate at which the burst frequency was increasing. Four ganglia began to decrease in duty cycle immediately, while the remaining 22 ganglia peaked at an average of 141% of the initial value. The ganglia reached an average of 13% of the initial duty cycle at the crash temperature.

The general decrease in burst duration was correlated with a decrease in the number of spikes per burst ($t(26)=0.8197$, $p<0.0001$), as in the semi-intact heart. The number of spikes per burst followed the same pattern as burst duration: it increased with increasing temperature, and then peaked and decreased at higher temperatures. The number of spikes per burst reached an average of 160% of the initial value at its peak, and returned to an average of 80% of the initial value at the crash temperature (Figure 15). Individual variation was particularly high for this parameter; some ganglia increased only a few percent from the initial value while others tripled in value before decreasing (Figure 15a).

The changes in burst duration were also correlated with increases in spike frequency ($t(26)=-0.9421$, $p<0.0001$). The spike frequency steadily increased over the entire temperature range, reaching an average of about 400% of the initial value at the crash temperature (Figure 16b). Individual ganglia reached spike frequencies ranging from 187% to 930% of the initial value before the ganglia crashed (Figure 16a).

3.4 Differences in the Crash Temperatures of Semi-Intact and ICG Preparations

The cardiac ganglion responded to increasing temperature in a stereotyped manner in both semi-intact and isolated preparations. While the crash pattern was the same when the ganglion was attached to the muscle and when it was isolated, the actual crash temperature differed by preparation (Figure 17). The isolated ganglion survived to a significantly higher

temperature (on average 4.6°C higher) than the semi-intact heart preparation ($t(21) = 6.289$, $p < 0.0001$). The ICG crash temperature was higher than the semi-intact crash temperature in 20 of the 22 hearts tested.

There was a possibility that the higher crash temperature seen in the ICG preparations could be explained by acclimation of the system to temperature perturbations. The nature of the dissection made it impossible to reverse the order of the preparations. To determine whether this was the case, control experiments were performed in which multiple temperature ramps were run on each semi-intact and isolated cardiac ganglion preparation (Figure 18). Semi-intact hearts subjected to multiple temperature ramps crashed at the same temperature each time. Ganglia isolated from these same hearts had increased crash temperatures that were maintained during multiple temperature ramps. We therefore concluded that the differences in crash temperature were due to the difference in preparation type and not to acclimation of the system to high temperatures.

3.5 Defacilitation of the Isolated Muscle at High Temperatures

The difference in crash temperature between semi-intact and isolated preparations could be due to temperature effects on the ganglion or on factors in the intact system. The semi-intact preparation contained multiple system components (cardiac ganglion, neuromuscular junction, and muscle tissue), all of which are likely dependent upon temperature in different ways. The system components were isolated using isolated cardiac ganglion and stimulated muscle preparations. In the stimulated preparation, the burst parameters were manually held constant, allowing for controlled analysis of temperature effects on the muscle and neuromuscular junction. The contraction amplitude of the muscle was not constant over the impulse train of 15

bursts. The system has previously been shown to exhibit significant facilitation over repeated bursts, until the amplitude reaches a plateau level (Anderson & Cooke 1971). The pattern observed at low temperatures resembled this facilitation; the contraction amplitude increased over the first few bursts, and then plateaued for the remainder of the burst train. At high temperatures, however, this pattern changed significantly. The contraction amplitude demonstrated the same initial facilitation, but the amplitude then decreased during the remaining bursts (Figure 19). The contraction amplitude reached its maximal value late in the impulse train at low temperatures, and reached its maximal value earlier in the impulse train at high temperatures. For example, around 10°C, the maximal contraction amplitude for the series of 15 bursts occurred around burst 12, whereas the maximal contraction amplitude at 30°C occurred around burst 3 (Figure 20a). In addition, the heart maintained a relatively constant contraction amplitude throughout the impulse train at low temperatures, but lost a significant percentage of the maximal contraction amplitude by the last burst of the impulse train at high temperatures. For example, only 6% of the maximal contraction amplitude was lost by the end of the impulse train at 10°C, but about 30% of the maximal contraction amplitude was lost by the end of the impulse train at 30°C (Figure 20b). Increasing temperature therefore produced defacilitation of contraction amplitude in the lobster cardiac muscle.

3.6 Stability in the Semi-Intact Heart and the ICG

There was some evidence that the muscle stabilized the system and helped to maintain regular, rhythmic bursting activity in the ganglion. The coefficient of variation for burst frequency is a measure of variability in bursting. This value increased significantly with increasing temperatures (significantly nonzero slope, $p < 0.0001$) in semi-intact hearts (Figure

21a), indicating that bursting became less stable and lost its rhythmicity at high temperatures. This increased variability was correlated with decreased muscle contraction amplitude ($r(24) = -0.7402$, $p < 0.0001$). In isolated cardiac ganglia, there was no change in the coefficient of variation in response to increasing temperature (zero slope, $p > 0.05$, Figure 21b). The coefficient of variation in semi-intact hearts was generally lower than the coefficient of variation in ICG preparations (except at very high temperatures). Therefore, the semi-intact heart showed greater stability than did the ICG, but this stability decreased with increasing temperatures. The ICG had higher baseline variability than the semi-intact heart, but did not become significantly more variable as temperatures increased.

Chapter 4

Discussion

A major goal in neuroscience is to understand the functioning of central pattern generator systems, which exist across many species and control a variety of behaviors. Although CPGs are able to function in the absence of sensory inputs, CPG systems do not exist naturally in isolation, and are subject to multiple intrinsic and environmental modulatory factors. CPGs often drive vital behaviors, such as respiration and heartbeat, and it is therefore important to understand how these behaviors may be altered in response to environmental changes. Temperature is an important environmental factor for nearly all species, and is especially pertinent for animals like lobsters, which are unable to regulate their body temperatures. Temperature is a ubiquitous modulator, able to affect physiological functioning at multiple levels. The lobster heart provides an ideal model system due to its relative simplicity, which allows for complete characterization of the effects of increasing temperatures on the cardiac system. A full study of this system may provide information applicable to understanding the function of more complex CPG systems in the future.

4.1 Crashing Behavior is Characteristic of CPGs

A previous study showed that the lobster heart can produce relatively stable output over the range of 2-20°C (Worden *et al.* 2006). The measure of cardiac output took into account both stroke volume (amount of hemolymph pumped through the heart, dependent on contraction amplitude) and stroke rate, or heartbeat frequency. Though the overall output remained stable, the underlying parameters were affected in different ways by increases in temperature: the frequency increased while the contraction amplitude decreased (Worden *et al.* 2006). At extreme

temperatures, however, the heart was unable to maintain its stable function. The “crash” behavior was therefore the disruption of system output due to the eventual dissociation between underlying parameters. Though compensation of these changes may have been possible over a limited temperature range, the system eventually reached a point where the underlying parameters had changed so dramatically from their initial states that they could no longer produce the patterned output (Rinberg *et al.* 2013). The definition of a “crash” also includes the ability of the system to regain function when temperature is returned to baseline (Tang *et al.* 2012). CPGs are notably resilient in this respect, indicating that the mechanism of “crashing” at high temperatures involves reversible processes. The definition of the “crashing” behavior seen in the lobster hearts of the current study thus provides clues for the underlying mechanisms through which temperature exerts its effects.

4.2 Acclimation Period Significantly Affected Crash Temperature

This study found a significant correlation between acclimation period and crash temperature for lobsters dissected during the fall and winter months. Previous work (Camacho *et al.* 2006) has shown that acclimation of lobsters to cold or warm temperatures can shift the thermal limits of cardiac functioning. Camacho *et al.* found that lobsters acclimated to 20°C can maintain heart function at higher temperatures than those acclimated to 4°C. An acclimation period of at least two weeks is ideal to allow metabolic functions to equilibrate, but significant results were seen after as few as three days for warm-acclimated lobsters (Camacho *et al.* 2006). Acclimation to a new temperature presumably activates long-lasting compensatory mechanisms, which then provide protection from more acute temperature changes to the system during experimentation.

The current results agreed with previous acclimation studies. Lobsters acclimated for longer periods of time during fall and winter months (September – February) crashed at higher temperatures in both semi-intact and ICG preparations. During this time period, the Maine ocean temperatures were colder than the laboratory tanks (National Oceanographic Data Center). Thus, the lobsters were acclimated to warmer temperatures in the lab, increasing their thermal cardiac limits and allowing them to maintain cardiac function at higher temperatures. These results also provided a clue as to the mechanism of acclimation. Either the processes involved in acclimation occurred at all levels of the system (since semi-intact and ICG preparations were similarly affected), or the processes involved were localized to the cardiac ganglion, with input from the muscle just shifting crashing behavior similarly in all cases.

There was no significant correlation between acclimation period and crash temperatures for lobsters dissected during the summer months. A possible explanation for the lack of acclimation effects was the moderate acclimation temperature used. While the Camacho *et al.* study used extreme temperature differences, our study acclimated lobsters to 10-12°C. This temperature range may be close enough to the temperatures encountered in the wild during the summer that the “acclimation period” may be extended to include some time before the lobsters were captured and brought into the lab. Therefore, our measures of acclimation period in the lab might underestimate the time spent at 10-12°C for summer lobsters, which would account for the lack of correlation found between acclimation period and crash temperature.

4.3 The Lobster Heart Responded in a Stereotyped Manner to Increasing Temperature

The isolated lobster heart responded reliably and consistently to changes in temperature. Generally, with increasing temperature, the burst frequency increased and the burst duration

decreased. Slightly below the crash temperature (where the ganglion lost all activity), the burst frequency trend reversed and frequency decreased quickly until it reached zero (no bursting) at the crash temperature. The shortened burst duration was correlated with a decrease in the number of spikes per burst and an increase in the spike frequency. These results are in agreement with previous studies on the effects of temperature on the intact lobster heart (Worden *et al.* 2006). The increase in the output rate, or burst frequency, is a common response of neural circuits to increases in temperature (Robertson & Money 2012).

The changes in burst duration were likely due to changes in the driver potential underlying cardiac ganglion firing. The driver potentials are dependent upon the balance of many different ion currents, including an inward calcium current and several outward potassium currents. The kinetics of the channels mediating each current may be differentially affected by increasing temperature, such that the timing of the currents may vary and change the shape and duration of the driver potential. The decreased burst duration was presumably correlated with a decreased driver potential duration. This effect could be caused by an increase in outward currents relative to inward currents, which would increase the repolarizing current and shorten the shoulder of the driver potential. The change in driver potential duration could be due to multiple relative changes in currents; for example, the inward current could decrease and the outward currents could stay the same, or the inward current could increase and the outward currents could increase at a higher rate. These different possibilities are important to consider when analyzing the changes in spike frequency. The increased spike frequency in response to increasing temperature is indicative of an increased depolarization from the driver potential. Therefore, the latter explanation (where both inward and outward currents increase) may be more

plausible. This hypothesis can be tested using intracellular recordings of motor neuron activity in ganglia exposed to temperature ramps.

Increasing temperatures also increased the amplitude of the extracellular spikes. The extracellular recordings measured the electrical activity of the entire anterolateral nerve, each of which contains axonal projections from four motor neurons (Hartline 1979). The extracellular signal therefore reflects the combined activity of multiple neurons. Spike amplitude is generally dependent upon axon diameter, but presumably this was not changing throughout the temperature experiments. There are two other possible explanations for this increase in amplitude, seen consistently across all hearts. The first is that the actual amplitudes of the action potentials increased with increasing temperature. The second is that, as temperatures increased, the firing of the motor neurons became more synchronized. The simultaneous firing of multiple motor neurons could then produce an additive effect, making the extracellular firing amplitude appear larger. The latter explanation may be contradicted by the increase in spike frequency. Unsynchronized, random firing of the motor neurons at low temperatures would result in high frequency, because consecutive spikes very close together in time could actually be coming from two different neurons. However, synchronization of the motor neurons at high temperatures would result in a lower frequency reflective of the actual burst frequency of a single neuron. In order to test these hypotheses, intracellular recordings would be needed of one or more ganglion motor neurons during a temperature ramp experiment.

Both the semi-intact heart and the isolated ganglion responded in similar manners to temperature increases. This suggests that the temperature dependence was due primarily to intrinsic properties of the cardiac ganglion. Interestingly, Worden *et al.* (2006) showed that isolated hearts and intact animals also had similar temperature dependencies. In contrast, another

study concluded that temperature dependence is instead altered by extrinsic modulation, because severing the cardiorespiratory nerves eliminated the changes in response to temperature (Jury & Watson 2000). This discrepancy may be explained by the very narrow temperature range used in that experiment (only 1.5°C up or down). Perhaps minute alterations of function are mediated through the cardiorespiratory nerve, but large changes in multi-parameter functioning are instead dependent upon direct temperature effects on the cardiac ganglion. These results suggest that it might also be useful to examine the combination of chemical modulators and temperature in this system as well.

4.4 The Contribution of Feedback to the Low Crash Temperature of the Semi-Intact Heart

Because the ganglion (nervous tissue) is particularly vulnerable to changes in temperature (Robertson & Money 2012), it seemed likely that the ganglion was the main site of temperature-dependent changes, driving similar patterns of response in the ICG and semi-intact heart. However, it was surprising to discover that the isolated ganglion continued to function at higher temperatures than the semi-intact heart.

We initially hypothesized that the presence of the muscle would provide stabilizing feedback to the system. Previous work in the Dickinson lab (Mara Chin-Purcell 2014) showed that the coefficient of variation for burst frequency increased as more feedback pathways were removed. For example, as compared to control, the burst frequency became significantly more variable when the dendrites that provide stretch feedback to the ganglion were cut, and even more variable when the heart was partially and fully de-efferented. The current study showed some support for stretch feedback stabilization. The contraction amplitude of the muscle in semi-intact preparations decreased as temperatures increased, and we concluded that the heart

provided less stretch feedback as temperature increased. This decreased contraction amplitude was correlated with an increase in the coefficient of variation for burst frequency in semi-intact hearts. Therefore, as contraction amplitude decreased and stretch feedback was lost, the heart became more unstable and lost bursting rhythmicity. In contrast, the ICG preparation contained no muscle, and therefore had no feedback to the ganglion. The ICG had a higher coefficient of variation than did the semi-intact heart even at low temperatures, suggesting that the presence of the muscle stabilized the intact system. The ICG had no feedback, so no feedback was lost as temperature increased, and correspondingly, there was no significant change in the coefficient of variation in response to increasing temperature. Taken together, these results suggest that the presence of the muscle in semi-intact hearts provided some stabilizing feedback to ganglion bursting activity.

These stabilizing effects related to stretch feedback, however, cannot account for the lower crash temperature of the semi-intact heart. A previous study showed that complete loss of stretch feedback to the ganglion resulted in increased variability in burst rate, and decreased burst frequency. However, in the absence of stretch feedback, the burst frequency fell only to about 50% of its original value in the intact system (Mara Chin-Purcell 2014). In the current study, the high temperatures caused the muscle to entirely stop beating and therefore to lose all stretch feedback. However, in this case, the complete loss of muscle contraction also entirely stopped the ganglion from bursting, rather than just decreasing the frequency. The relationship here between stretch and ganglion activity is complicated, and produces seemingly contradictory results. A way to resolve some of these questions would be to run a temperature ramp on a de-efferented heart and see if the crash temperature is comparable to the crash temperature of the isolated ganglion.

Another intriguing explanation for the semi-intact crash is the negative nitric oxide (NO) feedback system. NO is released by the heart muscle during contraction and diffuses to the cardiac ganglion, where it acts to slow the burst frequency. It has no effect on other ganglion parameters (e.g. burst duration and spike frequency) (Mahadevan *et al.* 2004). In the current study, burst frequency in semi-intact hearts actually increased over most of the temperature range due to thermodynamic considerations. However, at high temperatures, the frequency decreased quickly until the ganglion entirely crashed and ceased to burst. It is possible that temperature and NO had an additive effect at high temperatures, and combined to produce crashing behavior. At these high temperatures, the system may have been weakened due to the possible mismatch of channel kinetics controlling driver potential ion currents. In this weakened state, the NO feedback may finally have been able to overcome the increase in burst frequency and contribute to the rapid decline of the system near the crash temperature. This pathway could be tested by running a temperature ramp on a semi-intact heart in the presence of an NO synthase blocker, or by applying NO to the system at different temperatures to see if NO can promote crashing at a lower temperature.

4.5 Temperature Effects at the Level of the NMJ and the Muscle

Analyzing the effects of temperature on the contraction amplitude of the semi-intact heart is difficult due to the complex relationship between neural inputs and motor behaviors. Electrical neural signals are transformed into motor outputs at the neuromuscular junction, and this transformation depends upon many physiological factors. The pattern of contraction amplitude in response to increasing temperatures is therefore not easily described by a single change in ganglion functioning. Williams *et al.* (2013) showed that contraction amplitude in the lobster

heart is dependent upon the interaction of the burst frequency and the duty cycle (when spike frequency is held constant). This relationship is complex and nonlinear, and therefore burst parameters cannot be easily mapped into motor output. In the current study, contraction amplitude changed with increasing temperatures in a biphasic pattern; it initially increased and peaked around 12°C, and then decreased steadily over the rest of the temperature ramp. Interestingly, this biphasic pattern of amplitude change did not correspond to biphasic changes in burst frequency or duty cycle; frequency only increased and duty cycle only decreased over the entire temperature range. These results cannot easily be mapped onto the neuromuscular transform predictions of contraction amplitude given by Williams *et al.* (2013) because spike frequency was not constant in the temperature experiments. We concluded that the changes in contraction amplitude are due to the complex interaction of changes in the ganglion burst parameters and changes in the physiology of the neuromuscular junction due to temperature.

The complexity of the neuromuscular junction was amplified in semi-intact preparations, because ganglion function changed in response to temperature. The stimulated preparation was used to isolate the effects of temperature on the muscle and NMJ while holding burst parameters constant. The patterns of stimulated muscle contraction amplitude changed in response to temperature, indicating that temperature exerts effects at the level of the muscle and NMJ directly as well as through changes in ganglion functioning. At low temperatures, the stimulated preparation showed initial facilitation of contraction amplitude followed by a plateau of consistent amplitude. This pattern is in agreement with previous studies on muscle facilitation over a series of bursts (Anderson & Cooke 1971). In contrast, at high temperatures, contraction amplitude showed initial facilitation, but it was followed by a significant decrease in contraction amplitude. During normal functioning, the neuromuscular junction must integrate facilitating and

depressing inputs to produce an appropriate motor response. The change in contraction amplitude pattern at high temperatures may therefore be explained by considering the balance between facilitation and depression at various temperatures. At high temperatures, there may be an increased depression component and decreased facilitation component to account for the decrease in contraction amplitude seen over the series of bursts.

The lower crash temperature of the semi-intact preparation (as compared to ICG preparations) may also be explained by considering the neuromuscular junction and muscle. Previous studies have shown that, of the entire CPG-effector system, the neuromuscular junction is the most sensitive to heat (Prosser & Nelson 1981). A study in bats showed that the functioning limits of the CPG-effector system used in hunting calls were based on the limits of the muscle itself (Elemans *et al.* 2011). The current study showed defacilitation of contraction amplitude at high temperature, suggesting that depression at the level of the muscle may be responsible for the relatively low crash temperature of the semi-intact system.

4.6 Conclusions

The current study provides a characterization of the effects of temperature on the lobster cardiac system. Temperature, a global environmental factor, exerts its effects on heart function mainly through changes in cardiac ganglion functioning, as the same patterns of change are seen in both the semi-intact system and in the isolated ganglion. Temperature also affects the muscle and neuromuscular junction, and factors at this level (e.g. defacilitation at the NMJ or NO released by the muscle) may account for the relatively low crash temperatures of the intact system.

Lobster hearts show remarkable similarity in crash patterns among individuals. Even so, there is a lot of individual variation in the absolute values of parameter functions, as well as some variation in how these parameters change with temperature. These results may reflect the different underlying parameters that can exist in individuals (Goaillard *et al.* 2009, Marder 2011, Marder & Goaillard 2006, Prinz *et al.* 2004). Different combinations of underlying parameters can produce the same output, but may respond differently to system perturbations. High temperatures may therefore reveal the differences in underlying parameters by producing variable patterns of crashing and variable absolute crash temperatures. The current study provides a biological basis for understanding possible individual variation within a population.

More broadly, this study provides a basis for the study of more complex CPG systems. Though CPGs can continue to function without modulation, they are constantly exposed to a variety of modulatory factors from within the system and from the environment. The response and adaptation of CPGs to environmental perturbations is important for maintaining vital functions in the organism. An understanding of the mechanisms through which modulation may occur will allow for the study of larger, more complex CPG systems in other species, including humans, in the future.

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Figures

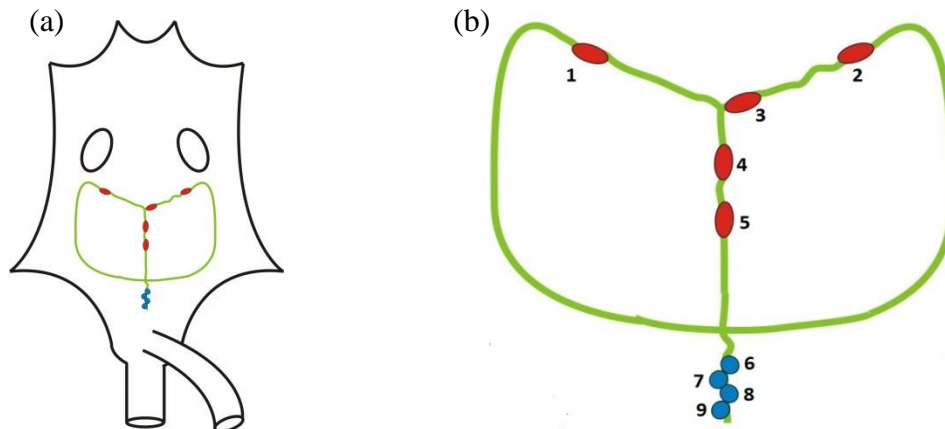


Figure 1. Schematic diagram of the lobster heart and cardiac ganglion. (a) Diagram of the heart showing the position of the cardiac ganglion within the muscle. (b) Larger view of the cardiac ganglion. Motor neuron soma are shown as red ovals (labeled 1-5) and interneuron soma are shown as blue circles (labeled 6-9). The interneurons produce spontaneous bursting and synapse onto the motor neurons. The motor neurons stimulate the heart muscle fibers through the neuromuscular junction and cause the heart to contract and pump hemolymph through the single-chamber heart.

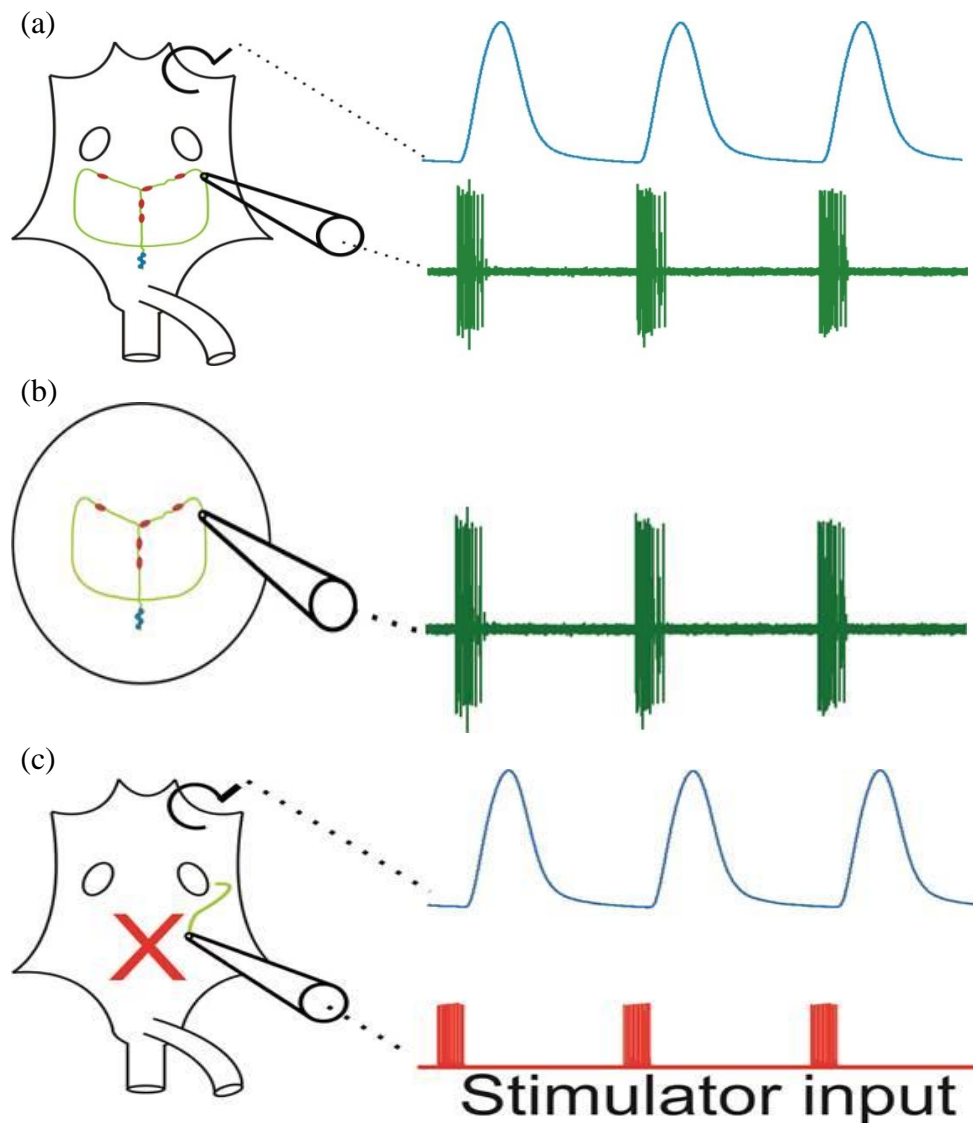
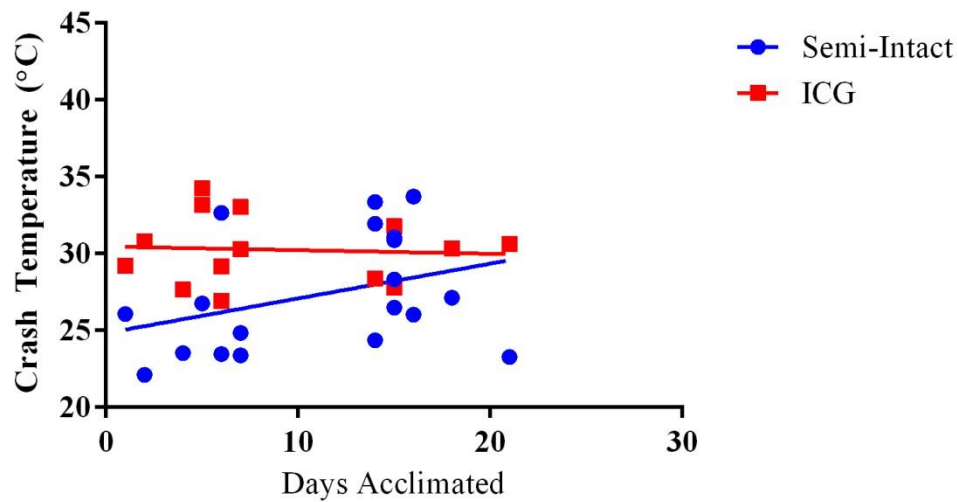


Figure 2. Schematic diagrams of the three preparation setups. (a) The semi-intact heart preparation contained the cardiac ganglion still attached to the muscle. Both the ganglion electrical activity (green trace, recorded with a suction electrode) and the muscular heartbeat contractions (blue trace) were recorded. (b) The isolated cardiac ganglion preparation contained only the ganglion after removal from the muscle. Ganglion electrical activity (green trace) was recorded using either a pin electrode within a Vaseline well or a suction electrode, as shown here. (c) The stimulated preparation contained only the heart muscle after removal of the ganglion (large red X). Externally-controlled current was injected through a suction electrode into the remaining anterolateral nerve ending to stimulate the muscle. The muscle contraction (blue trace) was recorded.

(a) June-August



(b) September - February

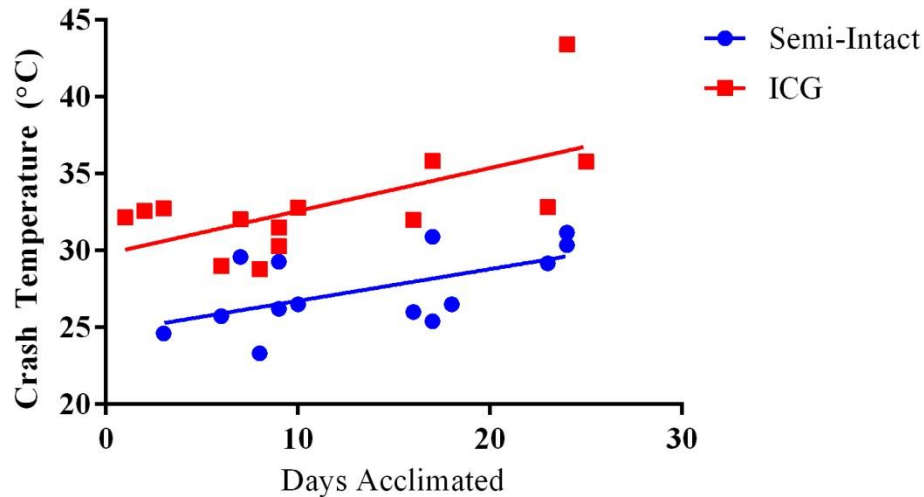


Figure 3. Crash temperature as a function of days acclimated. (a) There was no significant correlation between days acclimated and crash temperature for lobsters dissected June-August. The slopes of the best-fit lines are not significantly different from zero. (b) There was a significant correlation between number of days acclimated (at 10-12°C) and crash temperatures for lobsters dissected September-February. The slopes of the best-fit lines for both semi-intact and ICG preparations are significantly different from zero ($p < 0.05$).

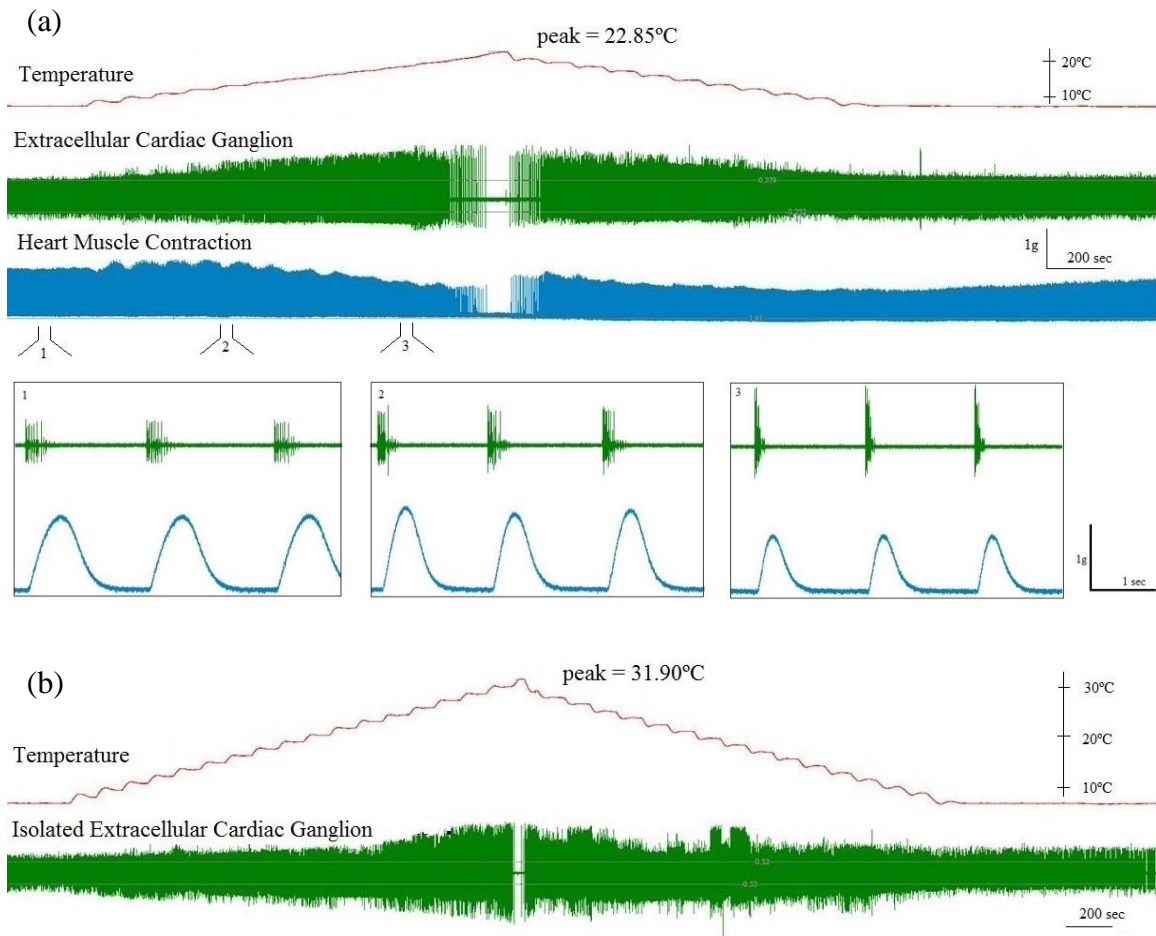


Figure 4. Condensed overviews of temperature experiments. Crashing behavior was defined as the complete loss of ganglion activity (and muscle contraction) for at least 30 seconds, with full recovery of function upon return to baseline temperature. (a) Semi-intact heart temperature experiment. The top trace (red) shows temperature, the middle trace (green) shows the electrical ganglion activity, and the bottom trace (blue) shows the muscle contraction. Boxes below show zoomed views of ganglion and muscle recordings of the regions indicated in the condensed overview. (b) Isolated cardiac ganglion temperature experiment. Top trace (red) shows temperature and bottom trace (green) shows electrical ganglion activity.

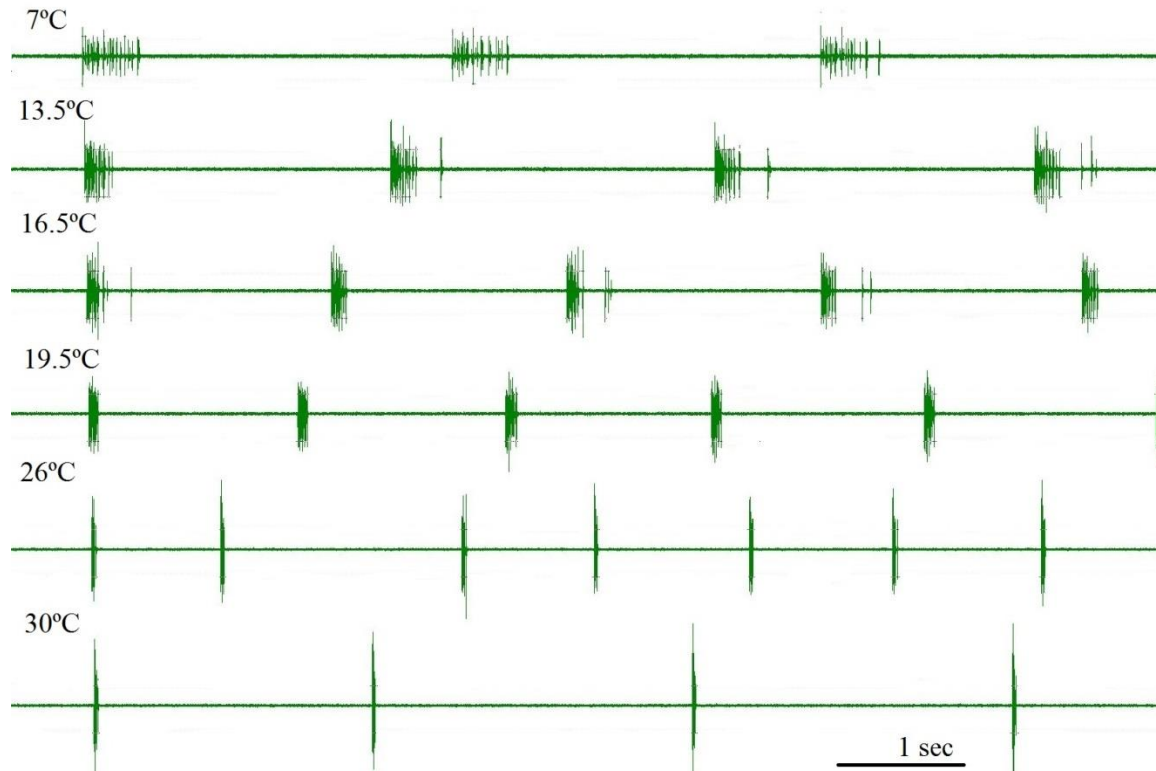
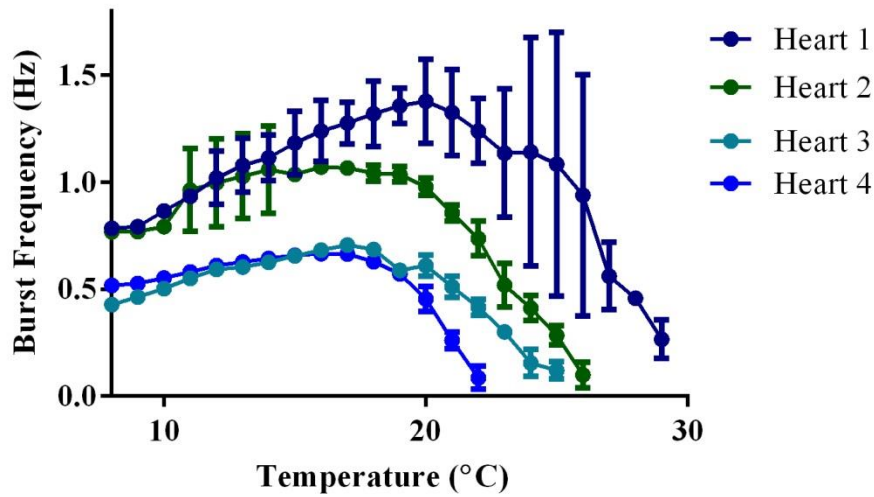
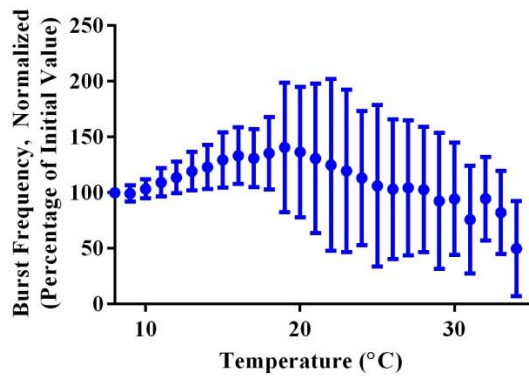


Figure 5. Sample motor nerve recordings from a single heart preparation at various temperatures. Changes in ganglion functioning may be qualitatively described. With increasing temperature, burst frequency increased, while burst duration and duty cycle both decreased. The decrease in burst duration was correlated with a decrease in the number of spikes per burst and an increase in spike frequency. The amplitude of the spikes also increased with increasing temperature. Before complete loss of bursting activity, the frequency became irregular and lost its rhythmicity. These patterns of change were common across all hearts analyzed.

(a)



(b)



(c)

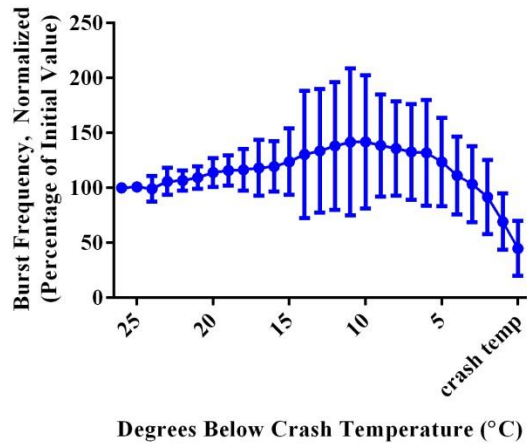


Figure 6. Burst frequency in semi-intact hearts. With increasing temperature, burst frequency increased to a certain point, then decreased quickly near the crash temperature (when the burst frequency = 0). (a) Burst frequency as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Averages and standard deviations were calculated in individual hearts using all burst data at each rounded temperature. (b) Combined data for all semi-intact hearts analyzed ($n=30$), shown on an absolute temperature scale. Data for each preparation were normalized so the average frequency at the starting temperature (usually 8°C) equaled 100%. Error bars show standard deviation. Averages and standard deviations for combined data were calculated using the individual averages at each rounded temperature. All 30 hearts remained functional at 22°C; at higher temperatures, hearts began to crash and the number of hearts represented in the average decreases. Twenty four hearts remained functional at 25°C, 9 hearts remained functional at 30°C, and 2 hearts reached 35°C before crashing. (c) Combined normalized data for all semi-intact hearts. The data were aligned so the crash temperatures match up for each heart; the x-axis shows degrees below crash temperature (=crash temperature – recorded temperature).

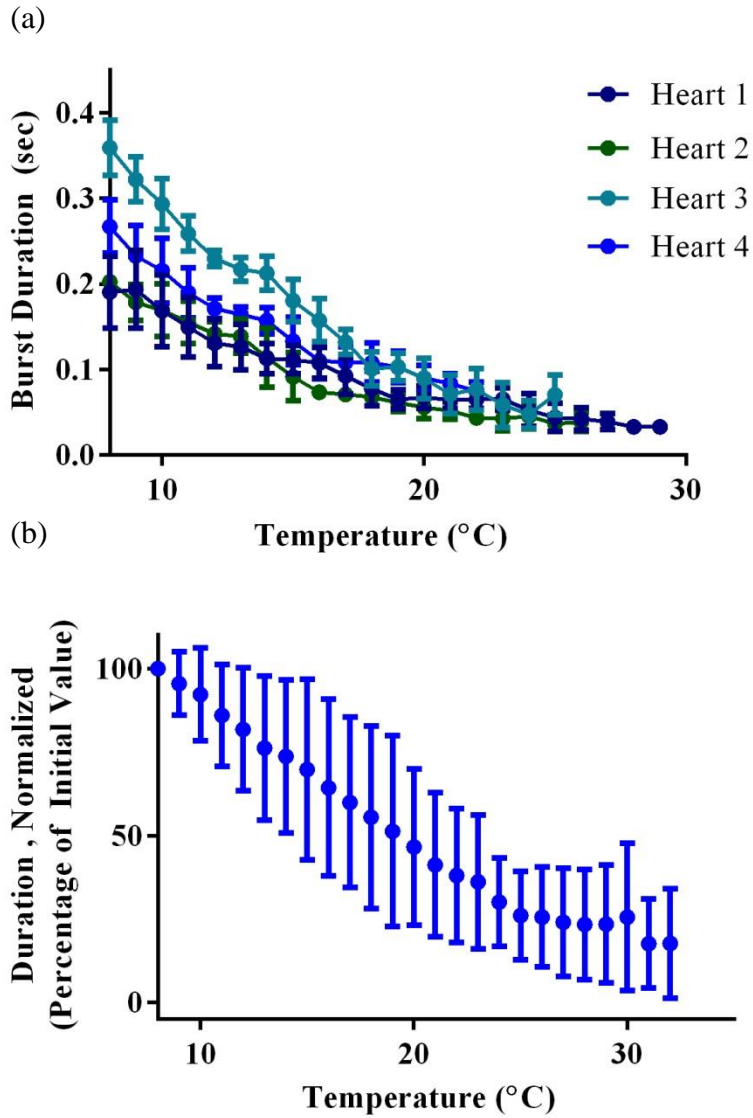


Figure 7. Burst duration in semi-intact hearts. Burst duration decreased over the entire temperature range. (a) Burst duration as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of hearts “1-4” corresponds to the same individual hearts shown in Figure 6. (b) Combined data for all semi-intact preparations analyzed ($n=30$). Data for each preparation were normalized so the average duration at the starting temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

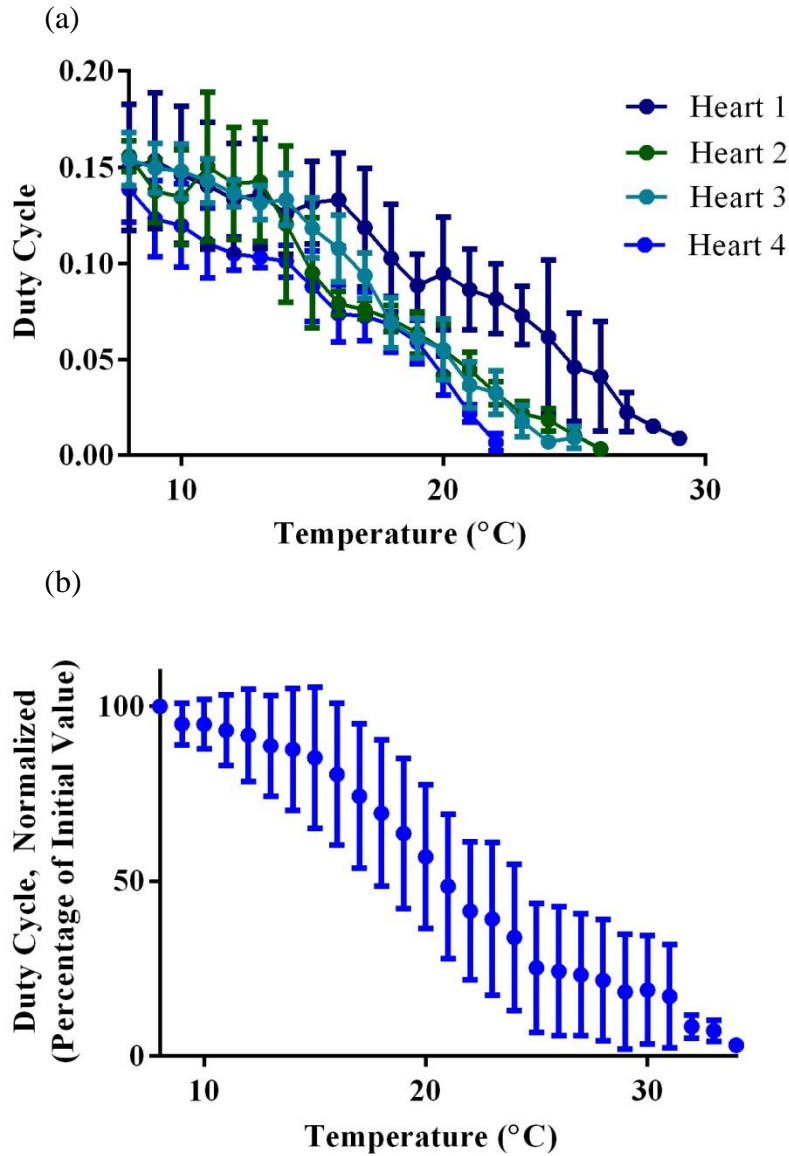


Figure 8. Duty cycle in semi-intact hearts. Over the first third of the temperature ramps, the changes in duty cycle were variable: duty cycle sometimes increased slightly, sometimes stayed constant, and sometimes began to decrease immediately. At higher temperatures, the duty cycle decreased in all hearts. (a) Duty cycle as a function of temperature for three individual heart preparations. Errors bars show standard deviation. Labeling of hearts “1-4” corresponds to the same individual hearts as in the previous graphs. (b) Combined data for all semi-intact preparations analyzed (n=30). Data for each preparation were normalized so the average duty cycle at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

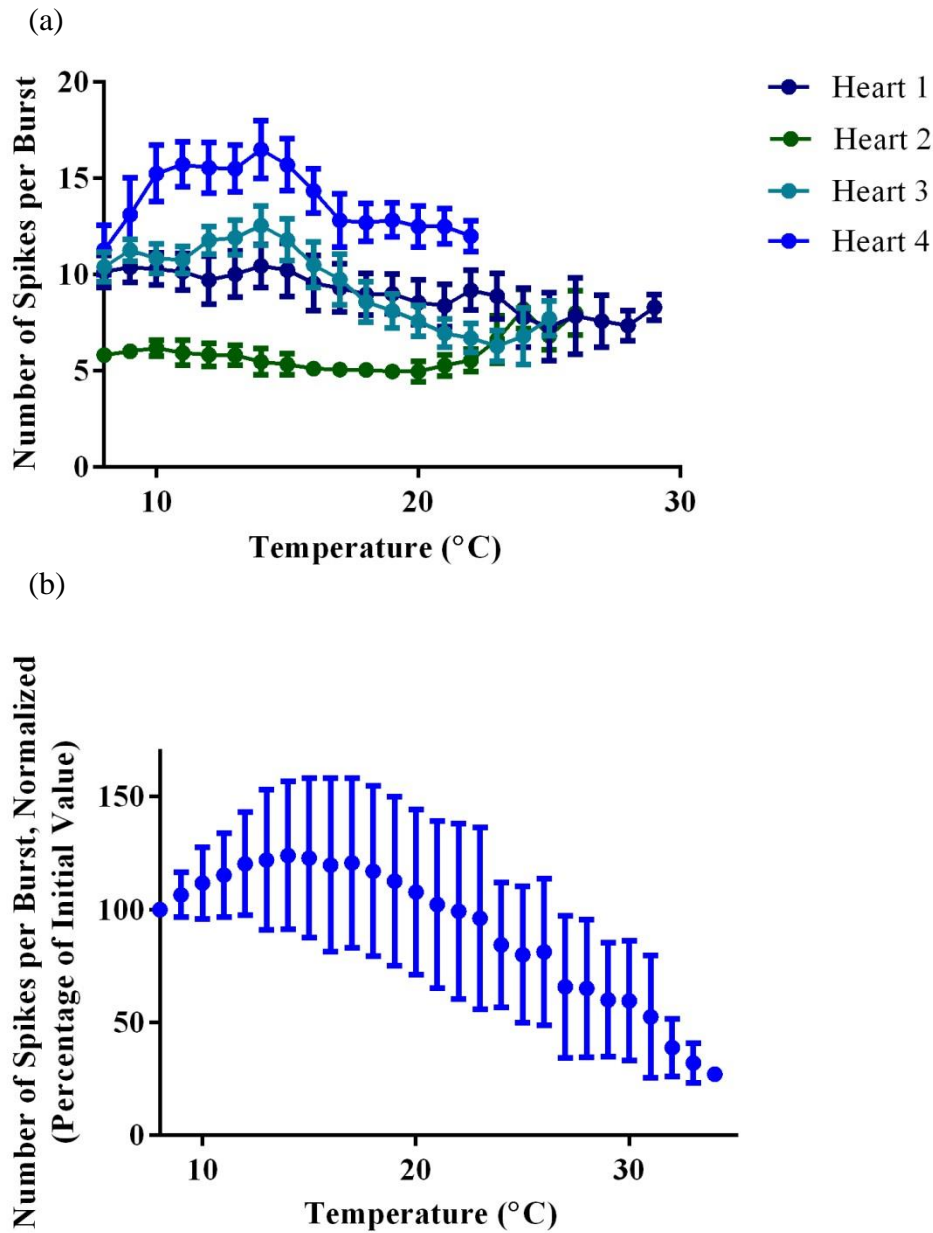


Figure 9. Number of spikes per burst in semi-intact hearts. The number of spikes per burst initially increased, then peaked around 14°C and decreased over the rest of the temperature ramp. (a) Number of spikes per burst as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of hearts “1-4” corresponds to the same individual hearts as in the previous graphs. (b) Combined data for all semi-intact preparations analyzed (n=30). Data for each preparation were normalized so the average number of spikes per burst at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

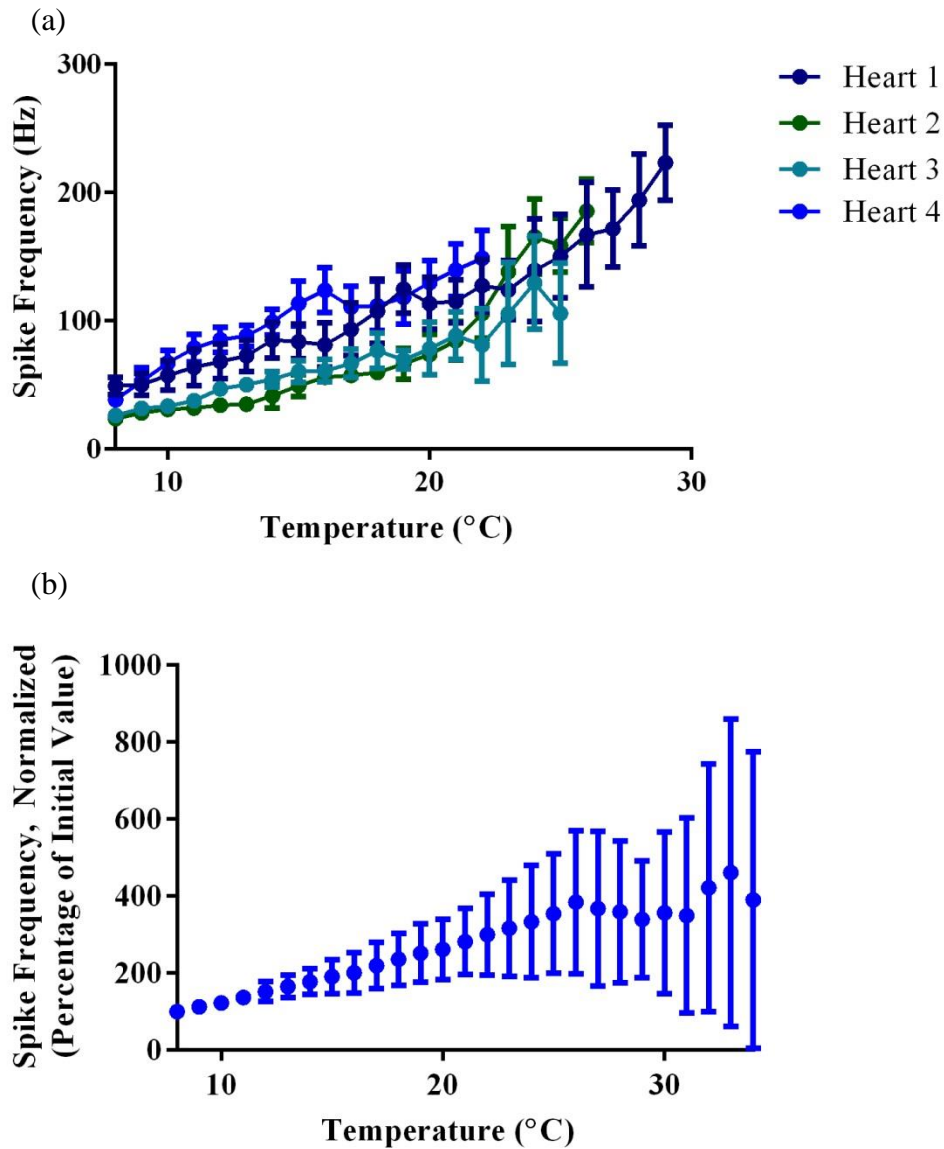


Figure 10. Spike frequency in semi-intact hearts. Spike frequency increased steadily with increasing temperatures. (a) Spike frequency as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of hearts “1-4” corresponds to the same individual hearts as in the previous graphs. (b) Combined data for all semi-intact preparations analyzed (n=30). Data for each preparation were normalized so the average spike frequency at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

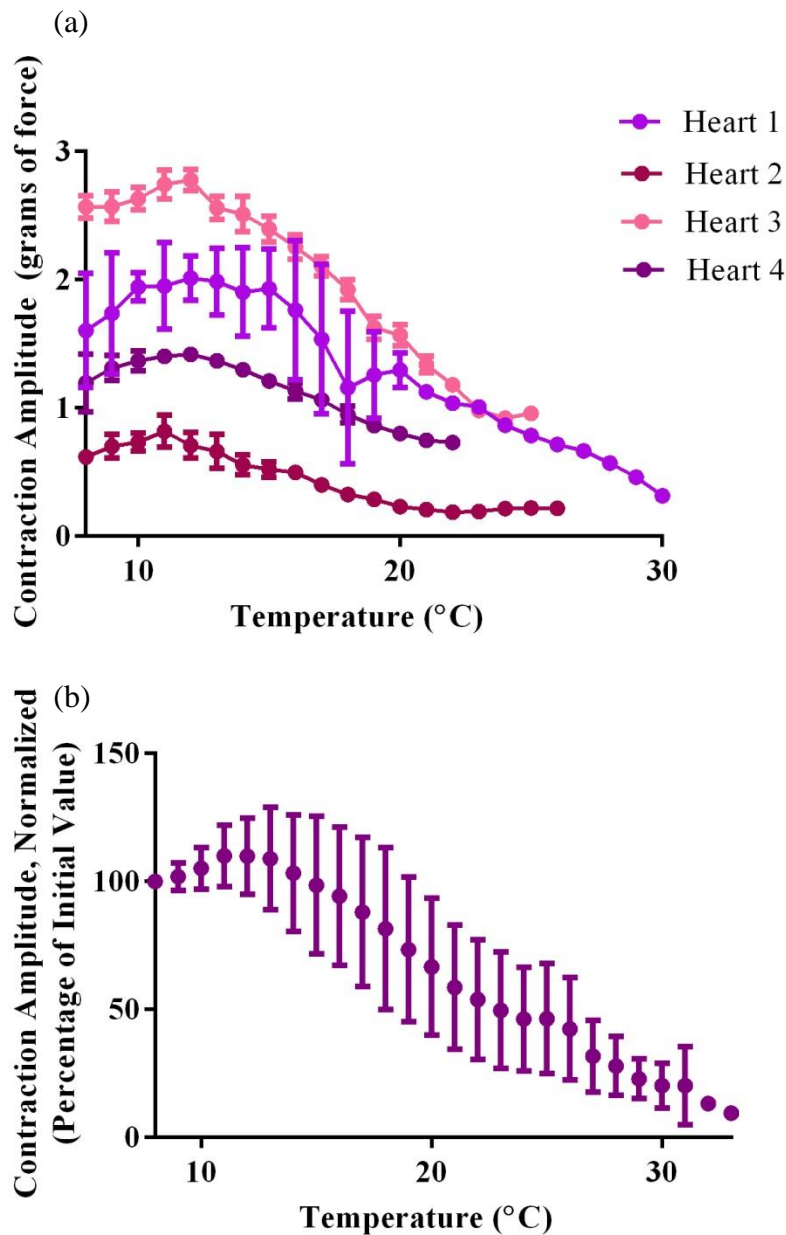


Figure 11. Contraction amplitude of muscular heartbeats in semi-intact hearts. The amplitude initially increased slightly, then peaked around 12°C before steadily declining over the rest of the temperature ramp. (a) Muscle contraction amplitude as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of hearts “1-4” corresponds to the same individual hearts as in the previous graphs. (b) Combined data for all semi-intact preparations analyzed (n=26). Data for each preparation were normalized so the average contraction amplitude at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

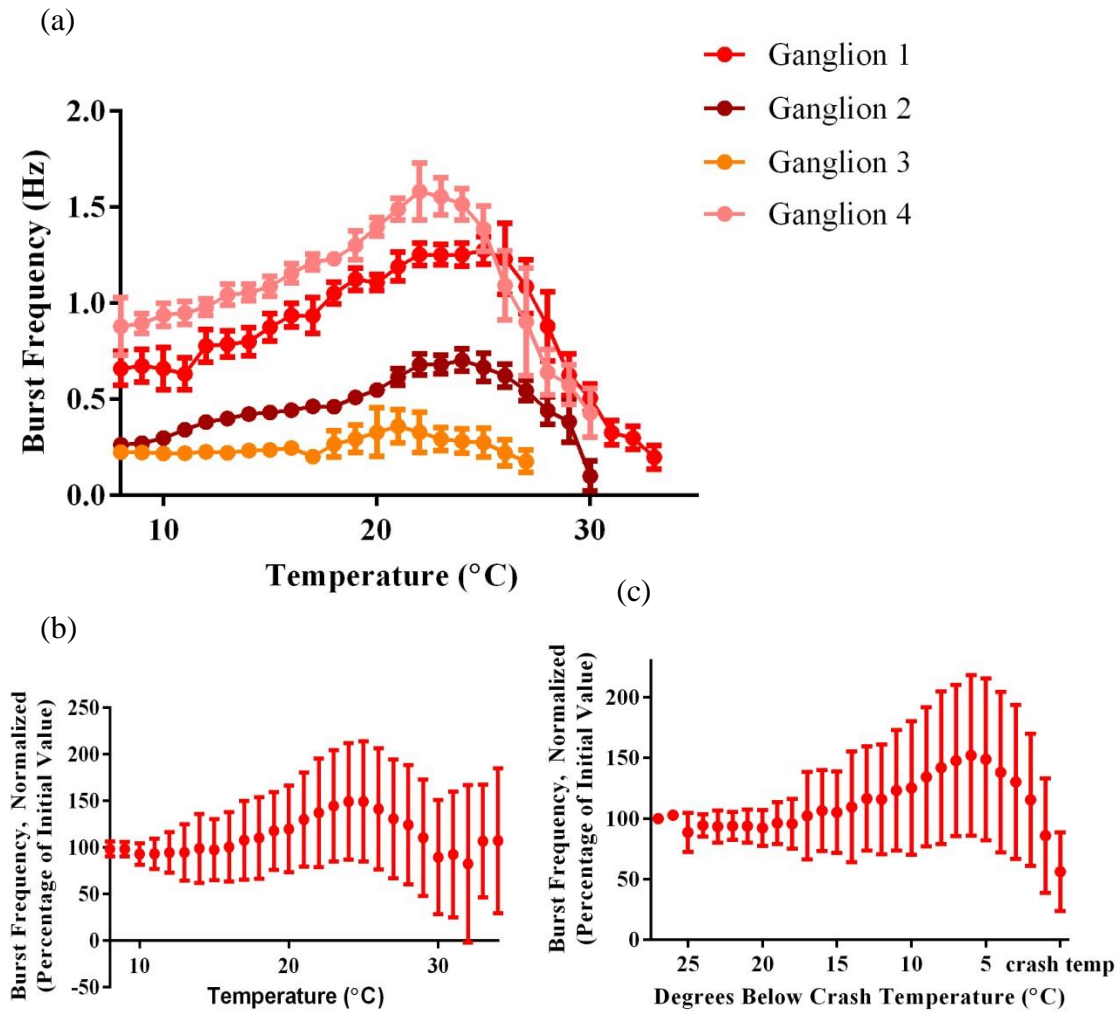


Figure 12. Burst frequency in isolated cardiac ganglia. Burst frequency increased with increasing temperature, then decreased rapidly at high temperatures close to the crash temperature. (a) Burst frequency as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of ganglia “1-4” was arbitrary and does not correspond to the same individual hearts as those shown in the semi-intact graphs. (b) Combined data for all isolated CG preparations analyzed (n=25). Data for each preparation were normalized so the average frequency at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation. All 26 ganglia remained functional at 27°C; at higher temperatures, ganglia began to crash and the number of ganglia represented in the average decreases. Twenty one ganglia remained functional at 30°C, 2 ganglia remained functional at 35°C, and 1 ganglion reached over 40°C before crashing. (c) Combined normalized data for all ICG preparations. The data were aligned so the crash temperatures match up for each heart; the x-axis shows degrees below crash temperature (=crash temperature – recorded temperature).

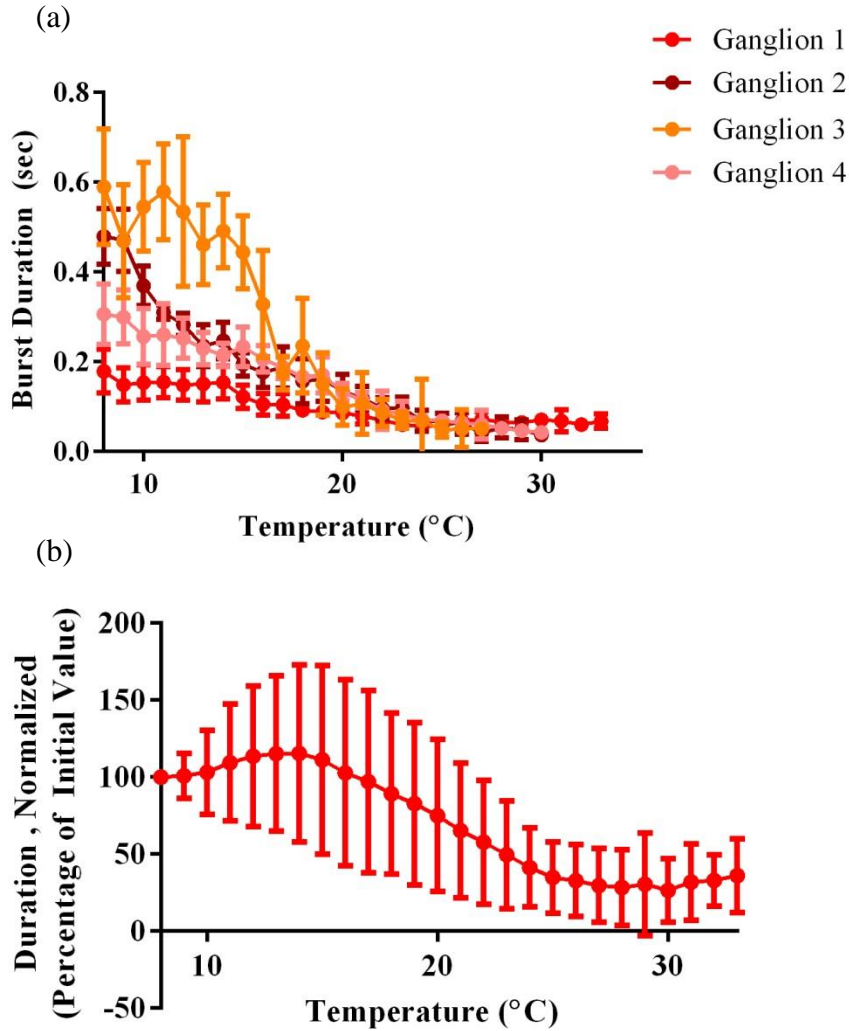


Figure 13. Burst duration in isolated cardiac ganglia. Burst duration increased slightly with increasing temperature, peaked at an average of 14°C, then decreased slowly at higher temperatures. (a) Burst duration as a function of temperature for three individual heart preparations. Errors bars show standard deviation. Labeling of ganglia “1-4” corresponds to the same individual ganglia shown in the previous ICG graph (Figure 12). (b) Combined data for all isolated CG preparations analyzed (n=25). Data for each preparation were normalized so the average burst duration at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

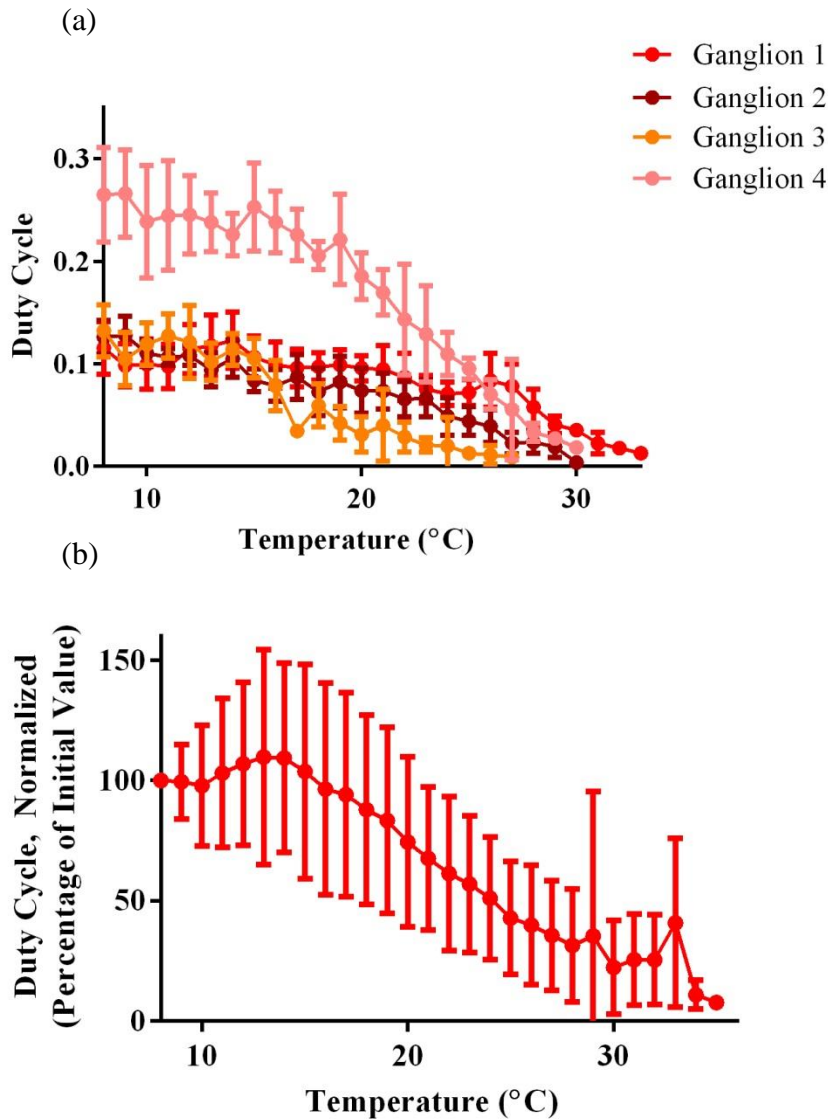


Figure 14. Duty cycle in isolated cardiac ganglia. Duty cycle followed the same pattern as the burst duration- it increased slightly at first and then decreased slowly over the rest of the temperature ramp. (a) Duty cycle as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of ganglia “1-4” corresponds to the same individual ganglia shown in the previous ICG graphs (Figures 12, 13). (b) Combined data for all isolated CG preparations analyzed (n=25). Data for each preparation were normalized so the average duty cycle at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

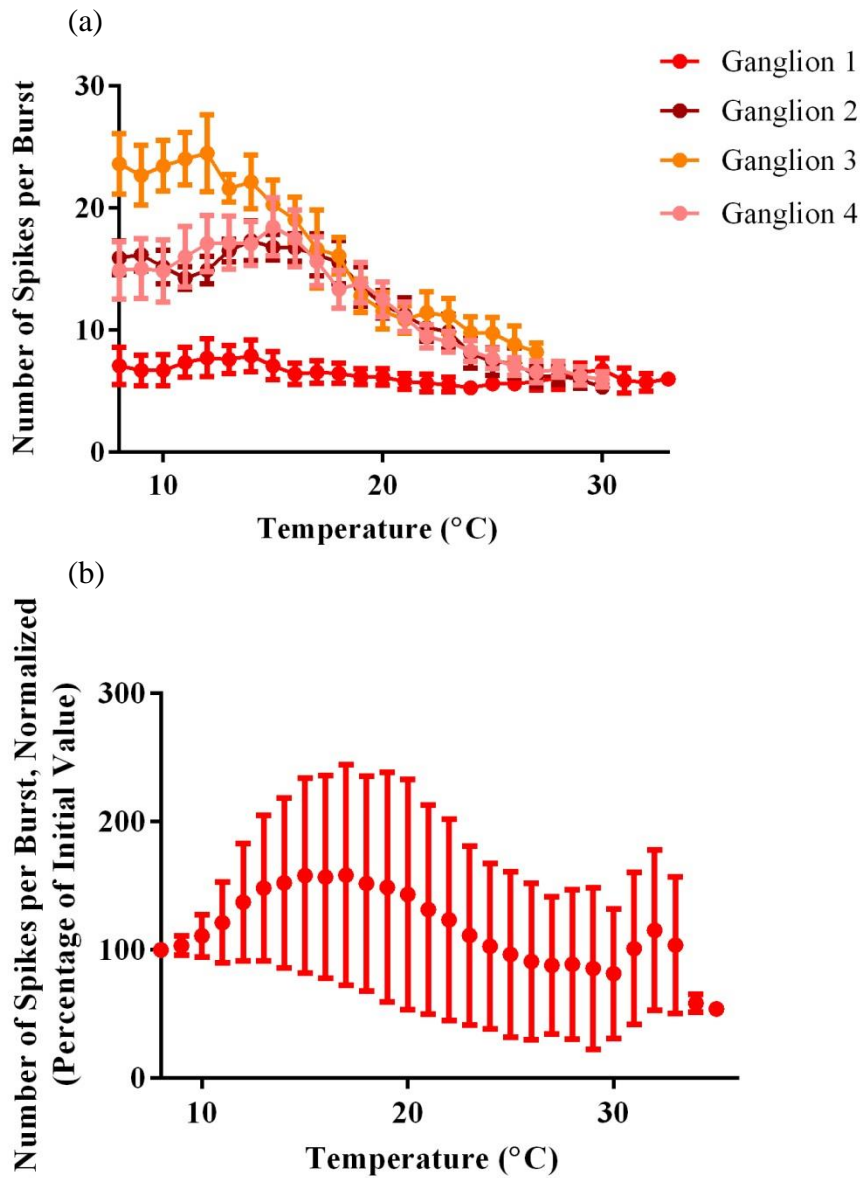


Figure 15. Number of spikes per burst in isolated cardiac ganglia. As temperature increased, the number of spikes per burst increased, peaked around 17 $^{\circ}\text{C}$, and then decreased to a value slightly below the initial baseline value. (a) Number of spikes per burst as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of ganglia “1-4” corresponds to the same individual ganglia shown in the previous ICG graphs (Figures 12-14). (b) Combined data for all isolated CG preparations analyzed (n=25). Data for each preparation were normalized so the average number of spikes per burst at the baseline temperature (usually 8 $^{\circ}\text{C}$) equaled 100%. Error bars show standard deviation.

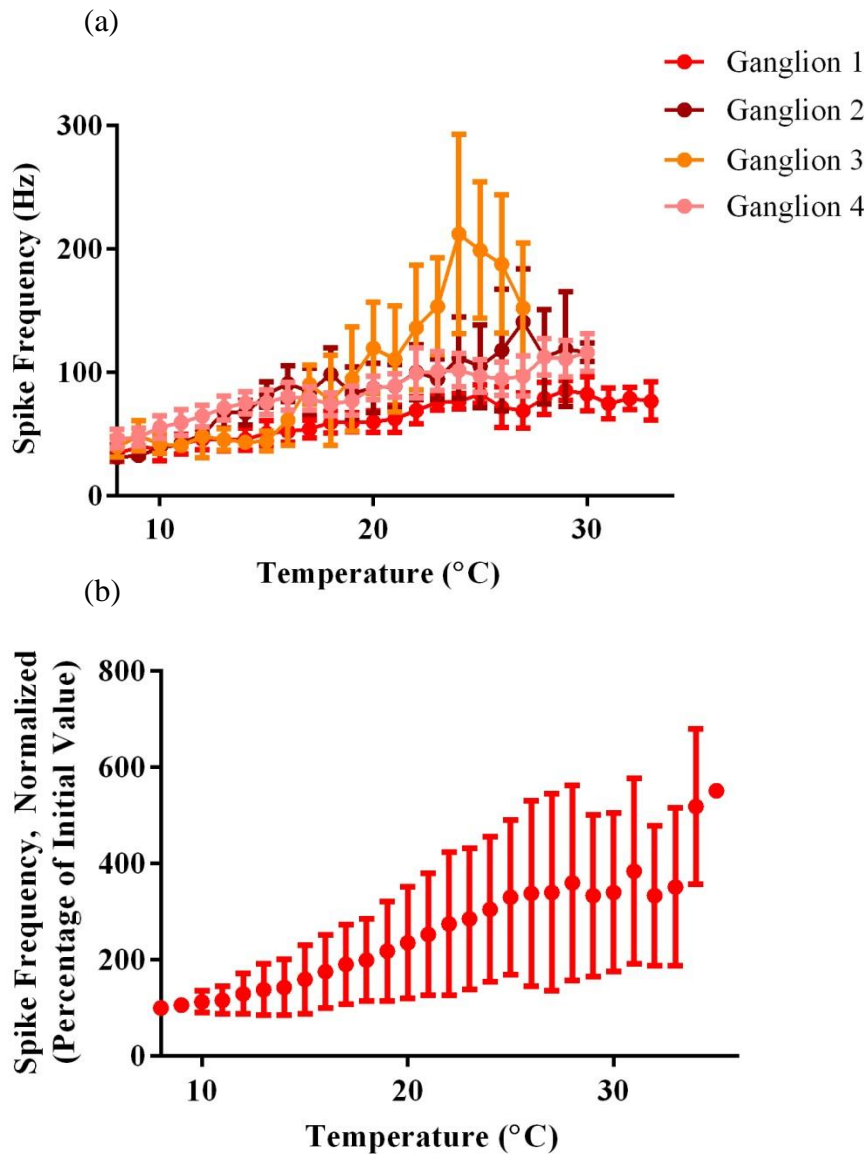


Figure 16. Spike frequency in isolated cardiac ganglia. Spike frequency increased over the entire temperature ramp. (a) Spike frequency as a function of temperature for four individual heart preparations. Errors bars show standard deviation. Labeling of ganglia “1-4” corresponds to the same individual ganglia shown in the previous ICG graphs (Figures 12-15). (b) Combined data for all isolated CG preparations analyzed (n=25). Data for each preparation were normalized so the average spike frequency at the baseline temperature (usually 8°C) equaled 100%. Error bars show standard deviation.

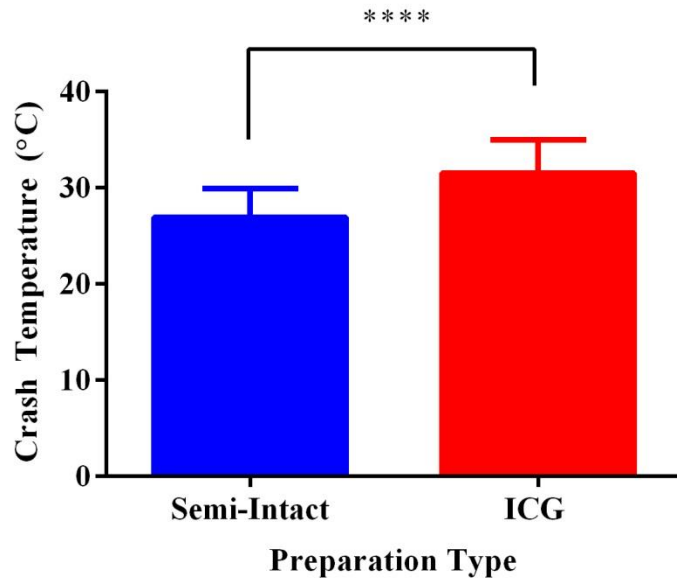


Figure 17. Comparison of semi-intact and ICG preparation crash temperatures. The ICG crashed at a significantly higher temperature than the semi-intact heart (n=22, **** = $p < 0.0001$). Error bars show standard deviation.

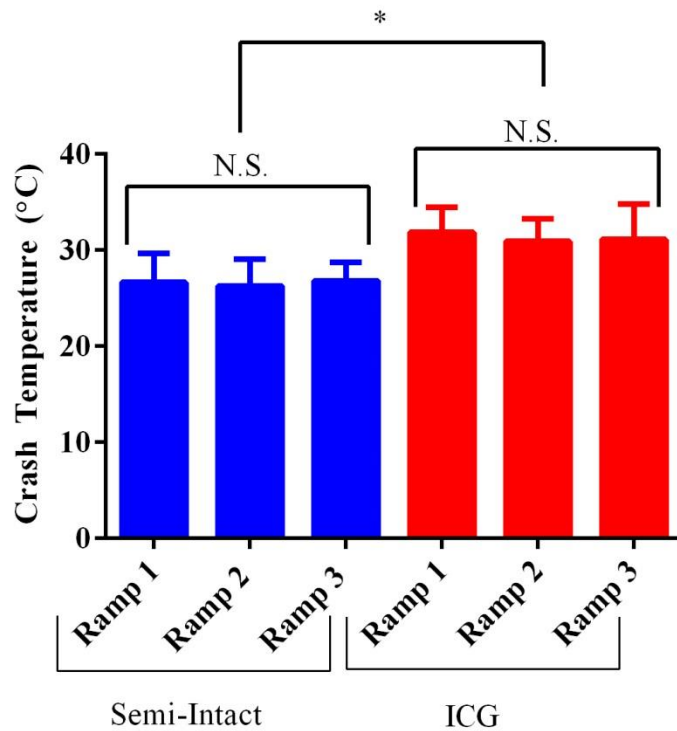


Figure 18. Short-term acclimation and crash temperature. Multiple temperature ramps were performed on several hearts to determine if the difference in crash temperatures of semi-intact hearts and isolated cardiac ganglia was due to preparation type or acclimation. There were no significant differences in crash temperatures among three consecutive temperature ramps performed on the same semi-intact heart. Nor were there significant differences among three consecutive temperature ramps performed on the same isolated ganglion. There was a significant difference between the average semi-intact and the average ICG crash temperatures, which was in agreement with the results from Figure 17 ($*=p<0.05$, NS = not significant). Error bars show standard deviation. These control experiments indicate that the differences in crash temperature between semi-intact hearts and ICG were due to true differences in preparation type and not to acclimation of the system.

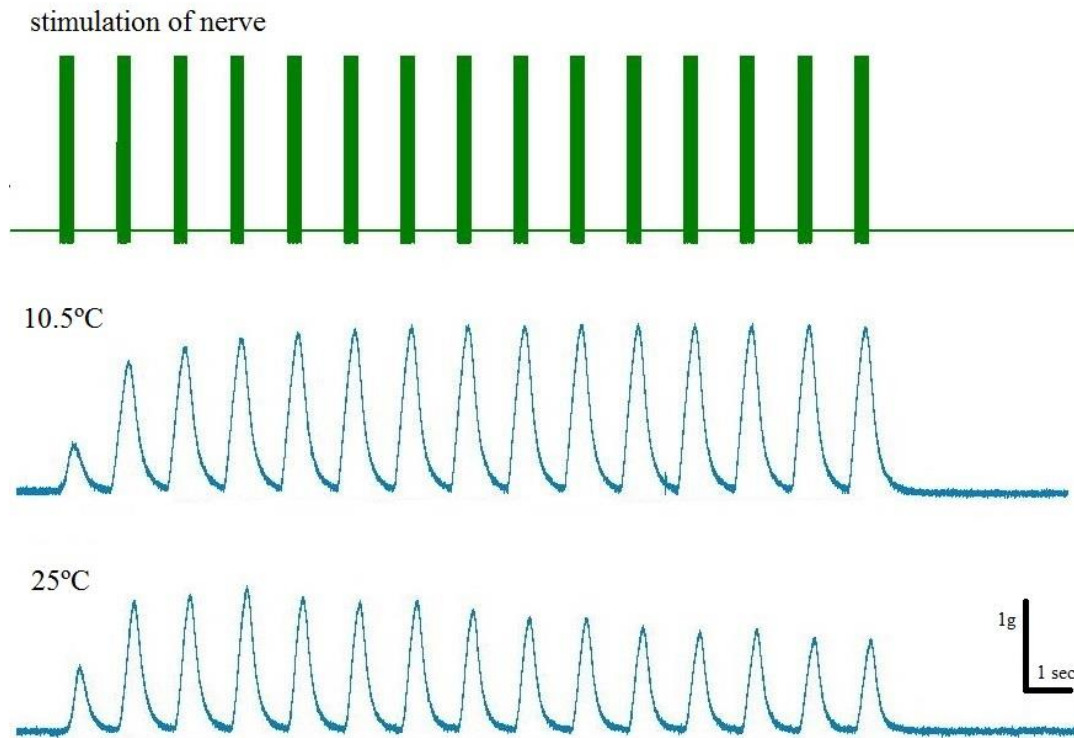


Figure 19. Muscle contraction in stimulated preparations. Impulses were applied to the nerve ending in trains of 15 bursts, with a 60 second break in between each train (one train is shown in the top trace). At low temperatures (10.5°C, middle trace), there was initial facilitation of contraction amplitude over the first few bursts, and then the amplitude remained relatively constant. At high temperatures (25°C, bottom trace), there was initial facilitation of amplitude followed by a large decrease in amplitude over the remaining bursts in the train.

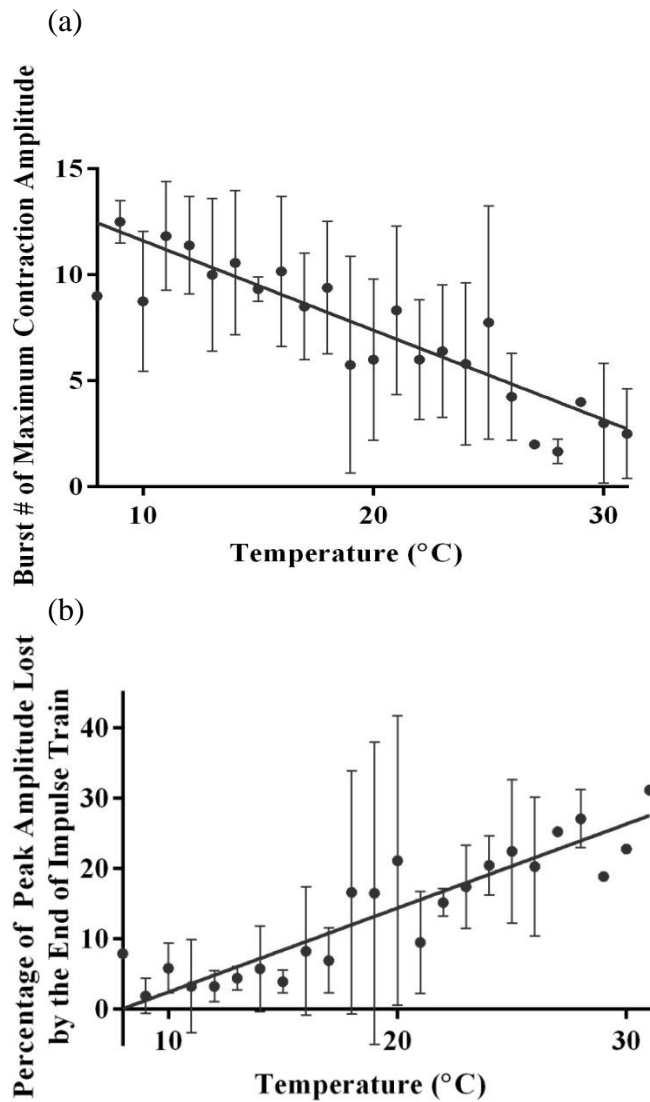


Figure 20. Contraction amplitude defacilitated in stimulated preparations. (a) Burst number (of the 15-burst train) where the contraction amplitude reached its maximal value. The maximal contraction amplitude was reached at a significantly later burst at high temperatures ($p < 0.0001$). Error bars show standard deviation. (b) Percentage of peak amplitude lost by the end of the impulse train. The muscle had significantly higher defacilitation of contraction amplitude with increasing temperatures ($p < 0.0001$). Error bars show standard deviation. Overall, at higher temperatures, the contraction amplitude peaked earlier and then decreased significantly; at low temperatures, the contraction amplitude showed initial facilitation and then remained at a consistent value throughout the burst train.

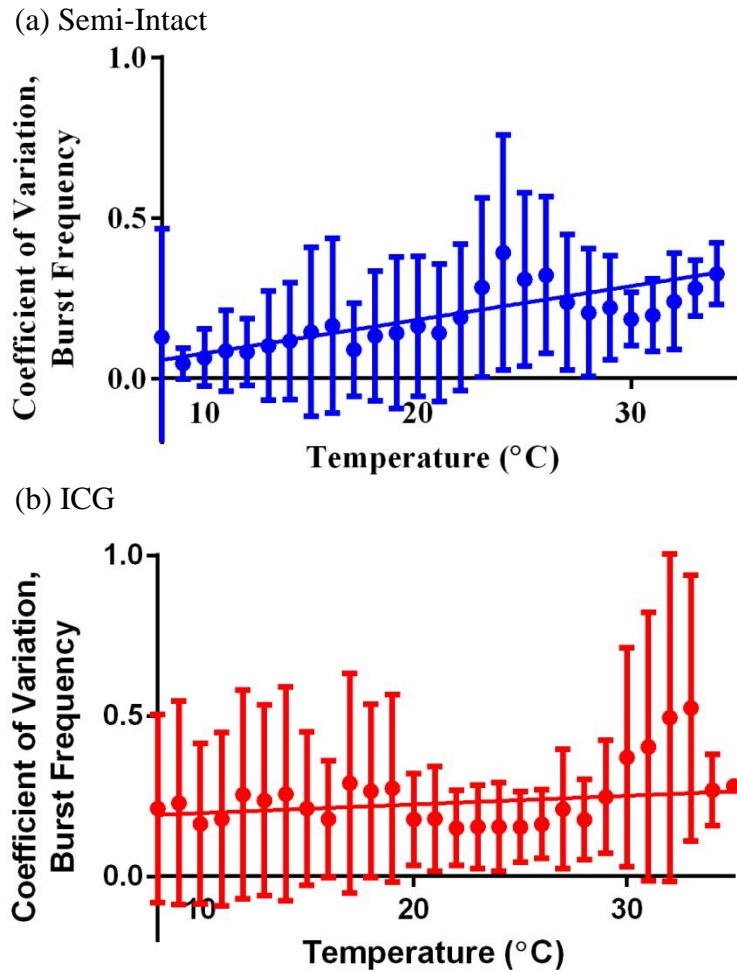


Figure 21. Coefficient of variation of burst frequency for semi-intact and ICG preparations. (a) The coefficient of variation increased with increasing temperature in semi-intact preparations. The best-fit line had a slope significantly different from zero ($p < 0.0001$). (b) The coefficient of variation was not dependent on temperature in ICG preparations. The slope of the best-fit line was not significantly different from zero. The coefficient of variation in semi-intact hearts was generally lower than the coefficient of variation in ICG preparations (except at very high temperatures).